REFERENCE ONLY

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Investigation of the Use of Cellular Gene Promoters in Murine Retroviral Vectors

A thesis submitted in partial fulfilment of the
requirements for the degree of
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by

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DECLARATION OF ORIGINALITY

I declare that this thesis is the result of my own work carried out at the Molecular Immunology Unit, Institute of Child Health, London. All works, analysis and graphic illustrations were carried out by myself, unless otherwise specified in the text.

Oliver Schwickerath

ABSTRACT

This study investigates the use of cellular promoters in the context of an enhancer-deleted murine retroviral vector suitable for application in gene therapy protocols for treating diseases that affect the myeloid lineage (Schwickerath *et al.*, 2004). Although the theory and practice surrounding enhancer-deleted and self-inactivating vectors has been known for some time, only recently have their attributes been considered to be of primary importance.

Our model system for the development of gene therapy has been chronic granulomatous disease/disorder (CGD), an inherited condition affecting the ability of neutrophils to kill invading pathogens, with potentially fatal consequences. The disease is caused by mutations in any one of several components referred to as gp91^{phox}, p67^{phox}, p47^{phox} or p22^{phox} which together with rac2 make up the NADPH oxidase complex present in neutrophils and other phagocytes (Casimir and Teahan, 1994; Goldblatt and Thrasher, 2000; Seger and Ezekowitz, 1994; Thrasher et al., 1992; Thrasher et al., 1993; Thrasher et al., 1994).

This project aimed to improve the transcriptional output and safety of retroviral vectors in myeloid cells, the cell type affected in CGD and to identify suitable tissue specific and inducible promoters for myeloid specific gene expression, which were employed for the expression of p47^{phox} (Rodaway et al., 1990; Teahan et al., 1990), the major cause of the autosomal recessive form of the disease.

To prove the functionality of our constructs *in vivo*, we made use of a p47^{phox} deficient mouse as a disease model for CGD, which lacks phagocyte superoxide production, thus reflecting an identical phenotype to that seen in human CGD (Jackson et al., 1995; Messina et al., 2002; Mitchison et al., 2005).

We conclude to have developed an enhancer-deleted retroviral vector that could provide the basis for a new generation of retroviral vectors with improved safety, by essentially retaining high titre and demonstrated transcriptional activity in myeloid cells, capable of reconstituting very high levels of oxidase activity, comparable to that obtained from normal cells.

DEDICATION

This thesis is dedicated to my late grandparents for their everlasting love, financial support and interest in the entire period of my lengthy education. I am deeply sorry, heartbroken and regret the missed opportunity to present the final reward to them.

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LIST OF ABBREVIATIONS

ψ	packaging signal (psi)
×	multiple times
°C	degrees centigrade celsius
μF	micro Farad (10^{-6}F)
μg	micro grams (10^{-6}g)
μl	micro liter (10^{-6}L)
3'	3 prime DNA terminus
5'	5 prime DNA terminus
ADP	Adenosine diphosphate
AmpR	ampicillin resistance gene β-lactamase
ATP	Adenosine triphosphate
BM	bone marrow
BMT	bone marrow transplant
Bp	base pair(s)
BSA	Bovine serum albumin
C/EBP-α	CCAAT/ enhancer-binding protein-α
CDNA	complementary DNA
CFU	colony forming unit
CGD	Chronic Granulomatous Disorder
CIP	Calf alkaline intestinal phosphatase
CMV	Cytomegalovirus
CMV IE	CMV immediate early gene
CsCl	caesium chloride
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
Dnase	Desoxyribonuclease
DNTP	Desoxythymidine triphosphate
E.coli	Escherichia coli
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetra acetic acid (disodium salt
EF1α	Elongation factor 1α
EGFP	Enhanced green fluorescent protein
Env	Envelope glycoprotein
ES	Embryonic stem cell
EtBr	ethidium bromide
F	Farad
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
Fig.	Figure
FITC	Fluroscein isothiocyanate
G	gram(s)
Gag	Group specific antigen protein
GALV	Gibbon ape leukaemia virus
GDP	Guanine diphosphate
GF	Growth factor
GTP	Guanine triphosphate
h	Hour(s)
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HEBS	N-[2-Hydroxyethyl]-piperazine-N'-[2-

	ethanesulfonic acid]
HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
HSC	Haematopoietic stem cell
Ig	Immunoglobulin
IL	Interleukin
IRES	Internal ribosome entry side
kb	Kilobase (10^3bp)
kDa	KiloDaltons (10^3Daltons)
LB	Luria Bertani medium
LTR	Long terminal repeat
luc	luciferase gene
M	Molar
mA	milli Amps (10^{-3} Amps)
MESV	Murine embryonic stem cell virus
MFI	Mean fluorescent intensity
mg	milli grams (10^{-3}g)
min	Minute(s)
ml	milliliter (10^{-3}L)
mM	milli Molar (10^{-3}M)
MoMLV	Moloney murine leukaemia virus (MLV)
MPSV	Myeloproliferative sarcoma virus
MRNA	Messenger RNA
MSCV	Murine stem cell virus
NADPH	Nicotinaminde adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
NE	neutrophil elastase
NF-κB	nuclear factor κB
ng	nanogram (10^{-9}g)
NK	Natural killer
nm	nanometre (10^{-9}m)
O_2	Oxygen
O_2^-	Superoxide
OD	Optical density
ORF	Open reading frame
Ori	Origin of replication
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PMA	phorbol 12-myristate 13-acetate
Pol	RNA dependent DNA polymerase
PolyA	Polyadenylation
RA	retinoic acid
RNA	Ribonucleic acid
Rnase	Ribonuclease
RNAse A	ribonuclease A
rpm	Revolutions per minute
RT	Room temperature / reverse transcription
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
sec	Second(s)
SFFV	Spleen focus forming virus
SSC	Saline sodium citrate
ssDNA	Single-stranded DNA
TAE	Tris/acetate/EDTA buffer
TB	Terrific Broth

TBE	Tris/borate/EDTA buffer
TE	Tris/EDTA
TEMED	N ;N ;N ;N ;-tetramethylethylenediamine
TEMED	1, 2-bis[Dimethylamino]ethane
Tris	Tris(hydroxymethyl)amino methane
Tris-HCl	Tris solution (pH-corrected by addition of HCl)
tRNA	Transfer RNA
U	Unit
UV	Ultraviolet
V	Volts
v/v	volume per unit volume
W	Watts
w/v	weight per unit volume
wt	Wild type
AB	Antibodicum
ATCC	American Type Culture Collection
No.	Number

1 Introduction

Haematopoietic stem cell transplantation from a fully matched donor offers only a possible cure for infants born with inherited immune deficiencies, who otherwise may not survive beyond the first years of life. Where a matched donor cannot be found, haploidentical transplants from parental donors may be suitable; however these are associated with greater complications and lower success rates. Hence an alternative therapeutic option based on gene therapy has been an intensive field of advanced research over the past 25 years (Cone and Mulligan, 1984; Stuhlmann et al., 1984), which just recently resulted in several clinical trials (Blaese et al., 1995; Bordignon et al., 1995; Cavazzana-Calvo et al., 2000b; Ott et al., 2006).

The fundamental basis of gene therapy is the successful introduction of a gene into somatic cells, followed by long-term expression of therapeutic levels of the required protein. Theoretically, diseases caused by known monogenetic defects, could be treated and potentially cured by the insertion and expression of a normal copy of the mutant gene in the host cells. With the ability to genetically modify haematopoietic stem cells, which have the potential for self-renewal, the tools are available for durable expression of therapeutic transgenes in all lineages of mature blood cells for the lifetime of the patient. In this context, autologous bone marrow transplantation combined with viral gene transfer has many potential applications for a wide range of blood diseases. This idea of gene therapy provides the basic framework for the future therapy of monogenic diseases (Brenner, 1996).

Although most clinical trials in gene therapy have been aimed at the treatment of various types of cancer, the most popular candidates for gene therapy are commonly the inherited single gene disorders, primarily cystic fibrosis, haemophilia, Duchenne muscular dystrophy, and in particular blood disorders, due to accessibility of bone marrow and therefore, the gene therapy targets, the stem cells. Hence, the second group of inherited diseases most commonly targeted has been severe combined immunodeficiency syndrome, like adenosine deaminase deficiency (ADA-SCID) and X-linked severe combined immunodeficiency (X-SCID) representing about 20% of all

trials for monogenic diseases carried out until today (Edelstein et al., 2004) and represents the only group in which gene therapy has shown any lasting and clinically meaningful therapeutic benefits (Cavazzana-Calvo et al., 2000b; Cavazzana-Calvo et al., 2001; Ott et al., 2006; Thrasher et al., 2006). In particular, chronic granulomatous disorder (CGD), an immune deficiency syndrome associated with defective neutrophil function has also proven a good candidate for gene therapy due to its significant morbidity and mortality as well as a detailed accumulated knowledge of the genetics and biochemistry of this disorder.

The work described in this thesis has been part of a programme of research focused on the development of gene therapy approaches to the treatment of CGD.

1.1 Background: Chronic Granulomatous Disorder (CGD)

Chronic granulomatous disease (CGD) is a rare inherited immunodeficiency condition that affects phagocytes of the innate immune system. The disease is characterized by a greatly increased susceptibility to severe bacterial and fungal infections, with the main pathogens being *Aspergillus*, *Burkholderia cepacia*, and *Staphylococcus aureus* that are threatening to people with CGD. The immunodeficiency results from a failure in the respiratory burst of the professional phagocytes (particularly neutrophils, as well as macrophages and monocytes, but also other non-phagocytic cells, including eosinophils and B lymphocytes) to produce superoxide ions (O_2^-) and their derivates, which protect cells from bacterial invasion (Heyworth *et al.*, 2003; Meischl and Roos, 1998; Roos, 1994; Thrasher *et al.*, 1994). The disorder derives from mutations in any of the 4 genes encoding the protein subunits of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (also referred to as the respiratory burst oxidase), the enzyme that generates microbicidal (and pro-inflammatory) oxygen radicals (Babior, 1999). The NADPH oxidase is the electron donor and catalyses a one-electron reduction of oxygen to form superoxide in stimulated normal phagocytes. Whilst superoxide (O_2^-) itself has little microbicidal activity, its derivates (e.g. hydrogen peroxide (H_2O_2), hypohalous acids, and hydroxyl radical ($^{\bullet}OH$)) are toxic and potent microbicides that are essential for killing many invading microorganisms. In contrast, phagocytes of

CGD patients are characterised by an absence or in some cases very low level of intracellular superoxide production (Noack *et al.*, 1999). Affected individuals consequently develop recurrent, sometimes fatal, bacterial and fungal infections, chronic granulomatous processes, and autoimmune disorders (Foster *et al.*, 1998). Overall, CGD has a minimum estimated incidence of approximately between 1 in 200000 and 1 in 250000 live birth and is normally diagnosed in infancy or early childhood (Winkelstein *et al.*, 2000).

1.1.1 Phagocytosis

The killing of pathogens by phagocytic cells of the blood, in particular macrophages and neutrophils, is an essential function of the innate immune response (Cohen, 1994).

Macrophages reside in tissues throughout the body and are particularly abundant in areas where infections are likely to occur and are the first phagocytic cells to encounter invading pathogens. The other major phagocytic cell type are the neutrophils, and also monocytes and eosinophils which are abundant in blood but are not present in healthy tissues. Neutrophils are rapidly recruited by chemotactic signals to sites of infection by activated macrophages as well as by molecules released by pathogens themselves, e.g. formylmethionine-containing peptides and accumulate together with monocytes and eosinophils at the site of infection to ingest invading microbes and fragments of damaged tissue by phagocytosis (Bokoch, 1995).

Phagocytosis is triggered by adherence of a particle to the surface of the plasma membrane of a phagocytic cell. Neutrophils and macrophages display an array of cell-surface receptors that enable them to recognise and engulf pathogens. These include pattern recognition receptors such as toll-like receptors (TLRs) and cell-surface receptors for the constant Fc portion of IgG antibodies produced by the adaptive immune system, as well as for the C3b component of complement. Other phagocyte receptors adhere to mannose-terminated oligosaccharides and fibronectin. Commonly, bacteria and viruses are coated (opsonised) or display one or another of these substances, like antibody IgG, C3b, fibronectin or mannose on their surface, which increase the rate of adherence and ingestion of the pathogen. Opsonisation is also used

for extracellular killing, such as a virus-infected host cell, a cancer cell, or pathogenic organisms which are too big to be ingested. In this case, the phagocytic cell binds to the opsonised body via its Fc receptors and empties the content of its lysosomes directly onto the surface of the antibody-coated target.

For smaller particles and intracellular destruction, ligand binding to any of these receptors induces actin polymerization at the site of pathogen attachment, and causes the plasma membrane of the phagocytic cells to surround the pathogen and engulf it in a large in a membranous vesicle, the phagosome (Figure 1). Once the pathogen has been phagocytosed, the phagosome migrates into the cytoplasma and fuses with lysosomal granules. The lysosomes carry a highly microbiocidal, acidic and digestive cocktail, a combination of lysosomal enzymes and oxygen radicals.

Figure 1: Schematic representation of phagocytosis by a macrophage

(1) A bacterium, which may or may not be opsonised, is engulfed by the process of endocytosis. (2) The bacterium is ingested in a membranous vesicle called the phagosome. (3) Digestive lysosomes (granules) merge with the phagosome and release their contents (proteases and oxygen radicals), and form a structure called the phagolysosome. (4) The killing and digestion of the bacterial cell takes place in the phagolysosome. (5) The macrophage egests debris while processing the antigenic components of the bacterium, which it returns to its surface in association with MHC II for antigen presentation to TH cells (adapted from www.textbookofbacteriology.net).

Microbicidial killing is mediated by the release of digestive products into the vacuole of the phagolysosome. These cytotoxic agents are proteolytic enzymes derived from lysosomal granules that have fused with the phagocytic vacuole, and toxic oxygen radical species generated by the membrane-bound NADPH oxidase complex.

Phagocytosis is accompanied by an increase in oxygen consumption described as the 'respiratory burst', which is caused by activation of the NADPH oxidase enzyme complex that catalyses the reduction of molecular oxygen (O_2) to superoxide (O_2^-) (Casimir and Teahan, 1994; Jones, 1994; Leusen et al., 1996; Thrasher et al., 1994). While superoxide is unstable at physiological pH, it rapidly dismutates in the presence of water to produces hydrogen peroxide (H_2O_2). Hydrogen peroxide is limited in its destructive capacity, but acts as a substrate for the generation of highly toxic oxygen-derived compounds, e.g. the anti-microbial agent hypochlorite ($HOCl^-$) by myeloperoxidase and additional reactive oxygen species, which can be generated from H_2O_2 and O_2^- in subsequent reactions, like hydroxyl radicals (OH^-) and nitric oxide (NO) (Rosen et al., 1995). In addition, proton consumption in the phagocytic vacuole following electron transfer causes a pH rise that is an important factor in pathogen killing (Segal et al., 1981). This increase buffers the acidic environment for optimal activity and function of many degradative lysosomal enzymes, which whilst stored at pH 5.0, function optimally at neutral pH.

Whereas macrophages generally survive this killing frenzy by egesting digested debris and inserting the microbial antigenic components into their plasma membrane for presentation to lymphocytes in the immunological response and continue to patrol tissues for other pathogens, neutrophils usually die and lyse, which form the distinctive tint of the pus that forms in acutely infected wounds.

1.1.2 The phagocytic NADPH oxidase

The NADPH-oxidase, which was first described in 1973 (Babior et al., 1973), is a phagosomal and plasma membrane-associated enzyme complex that is abundant in neutrophils, monocytes/macrophages and eosinophils, though low level expression has also been detected in B-lymphocyte populations (Maly et al., 1989).

1.1.2.1 Activation of the NADPH oxidase complex

The oxidase complex is dormant in resting neutrophils but rapidly assembled when cells are activated by a variety of inflammatory stimuli (Nauseef, 2004). Stimuli include the constant (Fc) region of pathogen-bound antibody, complement fragments generated following pathogen-antibody interaction, peptides generated by pathogen degradation, factors released by inflamed tissue such as neutrophil-activating factors NAP-1 and NAP-2, and bioactive lipids. Activation is mediated by surface receptor binding that stimulates heterotrimeric G protein dissociation, and triggers a variety of signal transduction pathways (Cockcroft et al., 1984; Cockcroft and Gomperts, 1985; Maridonneau-Parini et al., 1986). A number of artificial agents are also capable of stimulating oxidase activity including phorbol esters such as PMA.

1.1.2.2 Components of the NADPH oxidase complex

The NADPH oxidase complex is made up of six subunits. These are, five unique "*phox*" protein components, which can be separated into the membrane-bound redox centre of the oxidase: the flavocytochrome b_{558} which is arranged as a 1:1 heterodimer of two subunits, $p22^{phox}$ (α-subunit, 22kDa) and $gp91^{phox}$ (β-subunit, 91kDa) (Huang et al., 1995) and the soluble cytosolic factors: $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ plus the small *rho*-related GTP-binding protein p21*rac* (Rac1 and Rac2), which translocates to the membrane upon cell activation (Abo et al., 1994; Clark, 1999; Nauseef, 2004; Segal and Abo, 1993). The affix *phox* is an abbreviation for *ph*agocyte *ox*idase (Babior, 1999).

1.1.2.3 The membrane-bound redox centre: flavocytochrome b_{558}

Mutual interaction of the components is necessary for assembly of the mature NADPH oxidase complex. This was indicated by cells derived from most CGD patients who had $p22^{phox}$ and $gp91^{phox}$ missing or a molecular lesion to either subunit (Parkos *et al.*, 1987; Parkos *et al.*, 1989; Segal, 1987; Yu *et al.*, 1997).

Chapter 1: Introduction

The formation of $gp91^{phox}$ protein is subject to the incorporation of two non-identical haem groups for each heterodimer within membrane spanning α helices, one located near the inside, and the other towards the outside of the cell (Yu *et al.*, 1997). The binding site positions for the substrate NADPH, and for the electron carrier FAD (flavin adenine dinucleotide) have been shown to exist within the C-terminal region of the $gp91^{phox}$ glycoprotein, though $p67^{phox}$ has also been shown to contain an additional NADPH-binding site (Smith et al., 1996b).

The transfer of electrons across the cell membrane is mediated by the haem groups of the flavocytochrome b_{558}, which constitutes the entire electron transporting apparatus of the NADPH-oxidase. The midpoint redox potential of the assembled complex is sufficiently low (-245mV) to catalyse the formation of superoxide anions (O_2^-) by one-electron reduction of molecular oxygen (O_2) by transmembrane passage of electrons from NADPH to molecular O_2. (Babior, 2004; Nauseef, 1999; Nauseef, 2004).

$$NADPH + 2\,O_2 \leftrightarrow NADP^+ + 2\,O_2^- + H^+$$

This formation of superoxide is the precursor for the generation of potent oxidant compounds as described. Due to the observation that the electron transport can be sustained for several minutes, the existence of a charge compensating mechanism was discovered, with the membrane-spanning N-terminal region of the flavocytochrome been identified as the site of a charge-compensating H+ ion conductance during the respiratory burst and to be responsible for the regulation of the phagosomal pH. Its additional function is to neutralise harmful acidification of the cytosol and to buffer the phagosome for optimal activity of secreted proteolytic granule enzymes (Banfi et al., 1999; Henderson et al., 1995; Henderson et al., 1997; Henderson and Meech, 1999; Nanda et al., 1993; Nanda et al., 1994; Qu et al., 1994; Segal et al., 1981). More recently, it has been established that the compensating charge produced by H+ ions from the cytoplasm is further supported by K+ channels, which provide a vital self-regulating mechanism that is also required to elevate the vacuolar pH. Consequently, microbial killing is achieved through a combination of NADPH oxidase activity and proteolytic enzymes acting at neutral pH (Segal, 2005).

1.1.2.4 The cytosolic components of the NADPH oxidase

The soluble components of the NADPH oxidase complex, which are found in the cytosol of resting phagocytes as a multi-protein complex, are: $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ and the guanosine triphosphate-binding protein p21*rac*.

1.1.2.5 The cytosolic factor $p47^{phox}$

The $p47^{phox}$ protein was originally identified as a 47kDa phosphoprotein based on its absence from the neutrophil cytosol of patients with autosomal recessive chronic granulomatous disease (AR-CGD) (Segal et al., 1985). For the NADPH oxidase to function the $p47^{phox}$ component is absolutely essential. In resting cells, it has been shown that cytosolic $p47^{phox}$ is associated with equimolar amounts of $p67^{phox}$ and $p40^{phox}$ in the form of a 240kDa (Park et al., 1992) to 290kDa (Heyworth et al., 2003) multi-protein complex, but also exists in its free form (Park et al., 1994). On activation, this cytosolic complex is translocated to the plasma membrane where $p47^{phox}$ interacts with both subunits of the phagocyte cytochrome b_{558} (DeLeo et al., 1995a; DeLeo et al., 1995b; DeLeo et al., 1999; Heyworth et al., 1991; Nakanishi et al., 1992; Sumimoto et al., 2004; Takeya and Sumimoto, 2003).

The amino-acid sequence of $p47^{phox}$ has been identified to carry two *src*-homology region 3 (SH3) domains, which are known to mediate protein-protein interaction by binding to proline-rich sequences. The $p67^{phox}$, $p22^{phox}$ and $p47^{phox}$ amino-acid sequences contain proline-rich regions, and interactions with $p47^{phox}$ is thought to be mediated by SH3-proline binding (de, I et al., 1996; de, I et al., 1997; Leto et al., 1994; Sumimoto et al., 1996). In addition, the interaction of the $p47^{phox}$ protein with the cytochrome b_{558} is also mediated by a highly cationic region that interacts with the carboxyl-terminus of the $gp91^{phox}$ β-subunit (DeLeo et al., 1995a; Nauseef et al., 1993).

The $p47^{phox}$ function itself is regulated by serine phosphorylation. Neutrophil activation results in phosphorylation at multiple serine residues; and several species of $p47^{phox}$ phosphoprotein have been identified (Rotrosen and Leto, 1990). The phosphorylation of $p47^{phox}$ occurs in two stages, a cytosolic stage required for translocation of $p47^{phox}$, and a

membrane-bound stage. Membrane translocation requires phosphorylation at a single serine residue only (at position 379) (Faust et al., 1995). Phosphorylation induces a conformational change in the $p47^{phox}$ protein that enables the interaction with the flavocytochrome b_{558} by exposing a before hidden SH3 protein-binding domain (Faust et al., 1995; Park and Babior, 1997; Swain et al., 1997). The two most phosphorylated forms of $p47^{phox}$ are absent in the activated neutrophils of patients with the X-linked form of chronic granulomatous disease (X-CGD) patients who are lacking flavocytochrome b_{558} (Okamura et al., 1988), indicating that phosphorylation continues after membrane association. Evidence suggests that $p47^{phox}$ undergoes continuous rounds of phosphorylation and dephosphorylation in activated cells (Heyworth and Badwey, 1990).

1.1.2.6 The cytosolic factor $p67^{phox}$

The cytosolic component $p67^{phox}$ was initially identified by virtue of its absence from the neutrophils of patients with an autosomal recessive form of CGD in which $p47^{phox}$ protein is present (Nunoi et al., 1988; Volpp et al., 1988). Translocation of $p67^{phox}$ to the membrane is dependent on $p47^{phox}$ and is absent in the neutrophils of $p47^{phox}$-deficient AR-CGD patients (Heyworth et al., 1991). As described here previously, $p67^{phox}$ exists in the cytosol in combination with $p47^{phox}$ and $p40^{phox}$, due to the amino-acid sequence of $p67^{phox}$ containing a proline-rich region and two SH3 domains, which mediate the interaction with $p40^{phox}$ and $p47^{phox}$ (de, I et al., 1994).

1.1.2.7 The cytosolic factor $p40^{phox}$

The 40kDa cytosolic protein $p40^{phox}$ was identified by co-purification with $p67^{phox}$ in resting human neutrophils (Tsunawaki et al., 1994; Wientjes et al., 1993). In these cells $p40^{phox}$ is tightly associates with $p67^{phox}$ as a component of the 240-290kDa cytosolic activation complex (Tsunawaki et al., 1996) and binding to both, $p47^{phox}$ and $p67^{phox}$ has been demonstrated (Fuchs et al., 1995; Fuchs et al., 1996; Ito et al., 1996). During activation of the NADPH oxidase, the $p40^{phox}$ protein is complexed to $p67^{phox}$ and translocates to the membrane, which is mediated by $p67^{phox}$ (Dusi et al., 1996; Someya et

al., 1996; Wientjes et al., 1993), but $p40^{phox}$ is not required to reconstitute oxidase activity in recombinant cell-free systems (Abo et al., 1992; Lopes et al., 1999; Lopes et al., 2004; Park et al., 1997; Rotrosen et al., 1993).

Whilst there are several reports which show $p40^{phox}$ a possible stimulatory role in NADPH activation (Cross, 2000; Kuribayashi et al., 2002; Tsunawaki et al., 1996), other reports have shown $p40^{phox}$ in an inhibitory role (Sathyamoorthy et al., 1997; Vergnaud et al., 2000). Though more recently, $p40^{phox}$ has been indicated as a negative regulator through phosphorylation, which leads to an inhibitory conformation that blocks oxidase activation (Lopes et al., 2004).

In addition the role of $p40^{phox}$ in NADPH activation appears to be in the stabilization of $p67^{phox}$ and their initial assembly into the 240-290kDa activation complex, mediated through the $p40^{phox}$ amino-acid sequence containing a single SH3 domain that has been implicated in the interaction with $p47^{phox}$ (Ito et al., 1996; Wientjes et al., 1993) as well as a novel 'PC' motif that mediates the interaction with the SH3 domains of $p67^{phox}$ (Nakamura et al., 1998).

1.1.2.8 The GTP-binding protein p21*rac*

The activation of the electron flow from NADPH through the FAD and the haem groups of the flavocytochrome b_{558} is dependent on the presence of the small *rho*-related (guanosine triphosphate) GTP-binding proteins p21*rac* (Rac1 or Rac2, depending on the species and phagocytic cell).

The *Rho* family of proteins are low molecular weight GTP-binding proteins. Both members *rac*1, which is expressed ubiquitously and *rac*2 are expressed in phagocytic cells; while *rac*2 is restricted to myeloid cells and accounts for over 96% of total *rac* protein in neutrophils (Heyworth et al., 1994), and is less abundant in macrophages from which *rac*1 was originally isolated (Abo et al., 1991).

Rac protein has been found to be absolutely required for oxidase function in the cell-free system (Abo et al., 1991). In the cytosol of resting neutrophils, p21*rac* exists in an inactive GDP-bound form, and is maintained by association with the GDP-dissociation

inhibitor *rho*-GDI. On activation, the GDP-dissociation stimulator (GDS) facilitates the formation of active GTP-bound *rac* by dissociation of GDP and *rac* translocates to the plasma membrane (Abo et al., 1994; Abo and Pick, 1991; Heyworth et al., 1993; Kwong et al., 1993; Pick et al., 1993).

Both *rac*1 and *rac*2 are capable of reconstituting NADPH oxidase function in the cell-free system in the presence of $p47^{phox}$ and $p67^{phox}$ (Heyworth et al., 1993) and interaction between *rac* and $p67^{phox}$ has been reported (Diekmann et al., 1994), which is required for oxidase function and they may therefore translocate together (Ahmed et al., 1998; Prigmore et al., 1995; Ridley et al., 1992). In contrast, translocation of p21*rac*2 has been shown to occur in the absence of either $p47^{phox}$ or $p67^{phox}$, suggesting that alternative mechanisms exist for oxidase activation (Dusi and Rossi, 1993; Heyworth et al., 1994).

Whilst NADPH-oxidase activity is dependent on continued receptor occupancy, and continued association of oxidase components at the membrane, the termination of the response is not simply mediated by the release of $p47^{phox}$ and $p67^{phox}$ into the cytosol, which have been shown to remain in association with the membrane well after the burst has stopped (Dusi and Rossi, 1993). The phosphorylation state of the guanine nucleotide of the GTP-binding protein p21*rac* is the potent regulator of termination of NADPH-oxidase activity.

The role and function in NAPDH–oxidase activity of another small GTP-binding protein, Rap1A, which has been shown to co-purify with the flavocytochrome b_{558}, is unknown (Dinauer, 2005; Goldblatt and Thrasher, 2000; M'Rabet et al., 1998; Quinn et al., 1989).

1.1.3 Activation of electron transport

Naturally, activation of the 'respiratory burst' by complex formation of the components of the NADPH oxidase between cytosolic and membrane bound components is initiated by opsonised particles and other soluble inflammatory mediators during phagocytosis. A model of the phagocyte NADPH oxidase in its resting and activated assembled state is presented in Figure 2.

Figure 2: Schematic representation of the complex formation and activation of the phagocyte NADPH oxidase

Shown is a model of the NADPH oxidase activation ('respiratory burst'): **A**. In its resting state the multi-component NADPH oxidase co-exists as membrane-bound and cytosolic protein components, which requires assembly for activation. Two subunits gp91^{phox} and p22^{phox} form the membrane-bound flavocytochrome $_{b558}$, as a heterodimer. The cytosolic components p40^{phox}, p47^{phox} and p67^{phox} exist together as a ~240-290kDa complex, while in its inactive GDP-bound state, the small cytosolic GTP-binding protein *Rac* (also known as p21*rac*2) is bound to *Rho* GDP-dissociation inhibitor *Rho*GDI. **B.** Upon activation p40^{phox}, p47^{phox} and p67^{phox} become stably associated with the plasma membrane through interactions of p47^{phox} and the subunits of flavocytochrome $_{b558}$. This translocation process is accompanied by the release of *Rac* from *Rho*GDI and the phosphorylation of p47^{phox} (at multiple sites), p40^{phox} and p67^{phox}. Binding of the cytosolic components is associated with a conformation change of the flavocytochrome $_{b558}$, which is conducive to electron transport from NADPH to oxygen, via the flavin and haem redox centers in gp91^{phox} , to form O_2^- (red dashed line). The associated excess of oxygen consumption is known as the 'respiratory burst'. Termination of the reaction is controlled by hydrolysis of GTP bound to *Rac*, and dephosphorylation of oxidase components. The compartment labelled 'inside' is the cytoplasmic space; 'outside' refers either to the extracellular or phagosomal space. Adapted from (Heyworth et al., 2003).

1.1.4 Genetics and molecular Pathology of CGD patients

About two-thirds of all CGD cases are inherited in an X-chromosome linked fashion, hence termed X-CGD, and are caused by variety of different mutations in the gene (CYBB, Xp21.1) which encodes the $gp91^{phox}$ β-subunit of the flavocytochrome b_{558}.

The other third of CGD patients have been found to inherit the immunodeficiency in an autosomal recessive manner (AR-CGD), arising from mutations in the genes for $p22^{phox}$ (CYBA, 16q24), $p47^{phox}$ (NCF-1, 7q11.23), or $p67^{phox}$ (NCF-2, 1q25) (Clark et al., 1989; Ochs et al., 1999; Rae et al., 1998; Roos et al., 1996), while there has only been one rare case of an inhibitory p21*rac* mutation discovered in an infant boy associated with a human neutrophil immunodeficiency syndrome (Ambruso et al., 2000; Williams et al., 2000), but no molecular lesions resulting in CGD caused by mutations in the genes for $p40^{phox}$, Rap1a and *Rac* have been reported (Dinauer, 2005).

Noteworthy, the incident of $p47^{phox}$ deficiency has been found to be about five times more common than $p22^{phox}$ or $p67^{phox}$ deficiency, and mutations in NCF-1 gene ($p47^{phox}$) account for approximately 25% of all CGD cases (Casimir et al., 1992; Casimir et al., 1991; Chanock et al., 2000)

Protein affected	Gene	Chromosome locus	CGD Subtypes	Incident
$gp91^{phox}$	CYBB	Xp21.1	$X91^0$, $X91^-$, $X91^+$	~ 65%
$p22^{phox}$	CYBA	16q24	$A22^0$, $A22^+$	~ 5%
$p47^{phox}$	NCF-1	7q11.23	$A47^0$	~ 25%
$p67^{phox}$	NCF-2	1q25	$A67^0$	~ 5%

Table 1: Genetic subgroups of chronic granulomatous disease.

The mode of inheritance is indicated by X (X-linked) and A (autosomal recessive); the superscript symbols indicate wheather the level of affected protein is undetectable (0), diminished (-), or normal (+). X91+ and A22+ represent detectable but non-functional protein.

Mutations described in $gp91^{phox}$ deficient X-CGD patients show a high degree of heterogeneity, which include large and small deletions, point mutations and insertions,

errors at splice junctions, and mutations in the 5`upstream regulatory region of the CYBB gene, many of them family-specific (Noack et al., 1999; Patino et al., 1999; Rae et al., 1998; Rae et al., 2000). And while in most $gp91^{phox}$ deficient CGD patients no detectable levels of $gp91^{phox}$ protein have been found, in less than 10% X-CGD is caused due either non-functional or low levels of $gp91^{phox}$ protein (Goebel and Dinauer, 2003; Roesler et al., 1999; Roos et al., 1996).

In contrast, the majority of $p47^{phox}$ deficient CGD patients ($A47^0$ CGD) were found to have only a single common mutation (Casimir et al., 1991; Iwata et al., 1994; Roesler et al., 2000; Roos et al., 1996; Volpp and Lin, 1993) based on a dinucleotide deletion (ΔGT) at a GTGT repeat at the beginning of exon 2, which predicts a frameshift and premature stop codon at amino acid 51 (Casimir et al., 1991). Only in one $A47^0$ CGD patient who was heterozygous for ΔGT, was diagnosed for a single nucleotide deletion and remains the only non-ΔGT mutation reported (Volpp and Lin, 1993).

The discovery of the existence of a pair of 98% homologous $p47^{phox}$ pseudogenes (referred to as ψNCF-1), each of which contains the GT deletion and which are located in close proximity to the functional NCF-1 gene at the chromosome locus 7q11.23 (Gorlach et al., 1997), suggested that the predominace of the ΔGT mutation is caused by recombination events between the functional NCF-1 gene and its highly homologous pseudogenes (Roesler et al., 2000; Vazquez et al., 2001).

1.1.5 Treatment and current therapy of CGD

Since chronic granulomatous disease (CGD) was first described as a hereditary condition affecting neutrophil function in 1957 (Berendes et al., 1957), the prognosis for CGD patients has improved significantly over the past two decades. Classically, patients who suffer severe, recurrent bacterial and fungal infections are treated by antibiotic prophylaxis with trimethoprim-sulfamethoxazole, which greatly improved the survival rate of CGD patients (Weening et al., 1983). Administration of interferon-gamma (IFN-γ) has been shown to restore superoxidase activity in patients with X-CGD (Ezekowitz et al., 1988; Newburger et al., 1988) and found to be a safe and effective

adjunctive therapy to reduced the recurrence of life-threatening infections for many patients (Bemiller et al., 1995). However recombinant human IFN-γ therapy is not routinely available to all CGD patients, not only due to its high costs but also that it has not been equally efficient as large studies in the USA and Europe were able to demonstrate (Goldblatt and Thrasher, 2000). Therapy with corticosteroid had some beneficial with a few patients (Chin et al., 1987) and although the first attempts to treat CGD by bone marrow transplantation failed due to tissue rejection (Goudemand et al., 1976), successful treatment by allogenic granulocyte transfusions has been reported (Bielorai et al., 2000).

With recent advances in research and development for bone marrow transplantation (BMT), allogenic stem cell transplantation has been successful with several patients disease free for several years after transplant (Del, I et al., 2003). Particularly, somatic gene therapy in combination with BMT which overcomes the problems of allogeneic transplantation, and is not limited by supply of suitable donor material, the most promising gene therapy trial for CGD began in 2004 using retroviral vectors and has been the first to show a significant improvement in the clinical condition of the patients (Ott et al., 2006; Ott et al., 2007).

However, the overall success in this trial was found to be dependent on insertional activation of genes regulating myeloid differentiation, resulting in the clonal expansion of transduced cells. This augmented expression is thought to have arisen from the choice of retroviral vector employed, which was based on the spleen focus-forming virus (SFFV). This vector, contains a strong enhancer in the viral LTR used to drive the transgene and this was thought to be ultimately responsible for the insertional mutagenesis reported (Ott et al., 2006; Ott et al., 2007). Haematopoietic clones bearing vector insertions in specific loci became dominant in the myeloid blood compartment, suggesting that the clonal expansion had originated from an immature myeloid progenitor rather than from a stem cell (Ott et al., 2006; Ott et al., 2007).

As the recent death of one of the patients treated has shown, the risks of retroviral vector induced insertional mutagenesis are too serious that for gene therapy to become a routine treatment for CGD in the future, it will depend on the development of new vector systems which are as efficient but with added biosafety profiles.

1.2 Vectors for use in Gene Therapy

A large number of vectors have been identified with characteristics useful for gene therapy applications. These include non-viral methods: naked and complexed DNA; non-integrating viral vectors based on DNA viruses: adenovirus, adeno-associated viruses, and herpesviruses; and integrating viral vectors based on RNA viruses: onco-retroviruses (now termed gamma-retroviruses) and more recently, lentiviruses which are used in laboratory and clinic to achieve somatic gene transfer. Because of the particular properties each vector system has its own advantages and disadvantages, and are limited by definition of their application range depending on factors such as packaging capacity, host range, cell- or tissue-specific targeting, replication competency, genome integration, safety and duration of transgene expression (Table 2).

Owing to their well characterised genome, life cycle and possibility of generating vectors which do not express viral proteins and are free from replication competent virus, vectors based on the murine leukaemia virus (MLV), were the first to be developed and used in a human gene therapy trial in peripheral blood T lymphocytes of patients with adenosine deaminase deficient severe combined immune deficiency (ADA-SCID) (Blaese et al., 1995).

Subsequently, there has been an impressive increase of both the number of genes identified that are associated with human disease and the number of vector systems available for therapeutic gene delivery. While gamma-retroviruses require cell division for stable gene transfer, lentiviruses, adenovirus, adeno-associated virus, and herpes simplex virus can infect non-dividing cells.

The first gene therapy trial for cystic fibrosis (CF) employed recombinant adenovirus (rAd) vectors (Crystal et al., 1994), which was followed by the first use of recombinant adeno-associated virus (rAAV) (Flotte et al., 1996) and rAAV2 (Flotte et al., 2003) vectors expressing the cystic fibrosis transmembrane conductance regulator (CFTR) gene in CF patients; and an AAV vector expressing blood coagulation factor IX in haemophilia B patients (Kay et al., 2000). However, first generation adenovirus vectors provided poor long-term expression and induced dose-dependent immune and

inflammatory responses, which even led to the death of one patient with ornithine transcarbamylase deficiency (OTCD)(Raper et al., 2003). In contrast, recombinant adeno-associated viral vectors have been successfully applied in various tissues, including lung (Conrad et al., 1996; Flotte et al., 2003; Flotte et al., 2005), liver (Manno et al., 2006), muscle (Fisher et al., 1997; Kessler et al., 1996), brain (Janson et al., 2002) and retina (Acland et al., 2001). However, although AAV vectors mediate stable gene transfer to host cells without causing any significant innate immune response or insertional mutagenesis, they possess only limited packaging capacity of just 5kb and in general show lower levels of transgene expression than that seen with adenovirus.

Herpes simplex virus (HSV) based vectors are large and complex DNA viruses, which have been employed primarily for gene transfer to the nervous system (Mata et al., 2006), taking advantage of their ability of gene transfer and long-term expression in non-dividing cells, but have the potential to cause an innate and adaptive immune response.

Lentiviral vectors were first proposed as a carrier of an antisense oligonucleotide sequence targeted to regions of the HIV genome with the aim of treating HIV infection (MacGregor, 2001) and for gene therapy of mucopolysaccharidosis type IIIA (MPS IIIA) (Anson et al., 2007). In general lentiviral vectors are promising tools due to their capacity to package relatively large sequences (~9kb) and their ability to stably transduce of a broad range of different cell types, particularly non-dividing and dividing cells (Amado and Chen, 1999; Naldini et al., 1996). However, like with gammaretroviruses, there remain safety concerns regarding the non-specific nature of lentiviral integration into the human genome with their preference for insertion into gene sequences in active chromatin regions (Schroder et al., 2002) potentially increasing the risk of insertional mutagenesis.

The application of gammaretrovirus-based vectors in potential gene therapy approaches for treating disease is now well established (Miller, 1992; Palu et al., 2000; Takeuchi and Pizzato, 2000). In the haematopoietic system specifically, the virtues of low pathogenicity, acceptably high titre, and the ability to efficiently incorporate the vector genome into the host chromosome have made retroviral vectors the system of choice.

Vector	*Advantages*	*Disadvantages*	*Applications*
Viral Vectors			
Gamma - Retrovirus	well understood and characterised Highly efficient entry into mitotic cells Stable integration and gene transfer into host cell (8-12 kb) titer concentration and broad host range through pseudotyping	Safety concerns due to insertional mutagenesis with secondary malignancy Infection limited to dividing cells overexpression of transgene as well as gene silencing over time relatively low titer	ex vivo treatments, like ADA-SCID, X-SCID, CGD, familial hyperlipidemia; tumor vaccines; general marker studies
Lentivirus	Stable integration and gene transfer into host cells (<9 kb) Efficient infection of non-dividing cells	Safety concerns insertional mutagenesis with secondary malignancy and risk of replication competent HIV	broader target cell range than retroviruses, e.g. dentritic cells and hematopoietic stem cells; gene therapy application in HIV and mucopolysaccharidosis type VII (MPS)
Adenovirus	High titer Efficient entry into most cell types High level of expression low pathogenic potential Infection of non-dividing cells ability to package large transgenes with helper-dependent adenovirus (< 30kb)	Immunogenic, stimulating T and B cell responses, innate immunity tropism not well understood Vectors contain viral genes, Generation of replication-competent virus transient gene transfer long-term effectiveness poor	targetted in vivo gene transfer (no integration into host). Cystic fibrosis (CF) and short-term treatments for cancer, e.g. Mesothelioma and Colon cancer; cardiovascular and liver disease, e.g. Ornithine transcarbamylase deficiency (OTCD)
Adeno-associated virus (AAV): e.g. rAAV2	site specific integration minimal or no innate host immune response Infection of non-dividing cells with varying tissue tropisms durable transgene expression no human pathology, less immunogenic than adenovirus	Limited packaging size (< 5 kb) Delayed transgene expression in vivo lower expression levels than that seen with adenovirus inefficient integration into host genome	localised in vivo gene transfer, which requires long-term transgene expression: in lung, e.g. Cystic fibrosis (CF); brain, e.g. autosomal recessive Canavan disorder; retina, e.g. Leber congenital amaurosis (LCA); muscle, e.g. Hemophilia B, and Alpha-1 antitrypsin (AAT) deficiency
Herpesvirus (HSV)	High titer High efficiency of gene transfer to non-dividing cells Neurotropic	relatively complex potential for innate and adaptive immune response	Neurological diseases. brain tumors and colon cancer
Non-Viral Vectors			
Naked DNA and DNA liposomes	Easy to prepare No size constraints No viral genes High level of safety transient: no integration improved uptake with the use of liposomes	Inefficient gene delivery and uptake transient gene transfer	DNA vaccines: for topical and/or mechanical applications, including skin and vasculature; and liposome facilitated direct in vivo applications: metastatic cancer, cardiovascular disease, cystic fibrosis (CF)

Table 2: Advantages and disadvantages of different vector systems in use for somatic gene transfer and their application range.

Indeed, in terms of clinical usage for haematopoietic gene therapy, retroviral vectors have been used more widely and more successfully than any other (Aiuti et al., 2002;

Blaese et al., 1995; Brenner, 1996; Cavazzana-Calvo et al., 2000b; Kohn, 1997; Ott et al., 2006; Thrasher et al., 2006).

In spite of their popularity, problems with these vectors still exist. The possibility of oncogenic transformation of host cells as a result of random non-specific insertion of retroviral DNA into the host chromosome has always been a concern. In addition their inability to efficiently transduce quiescent cells, transcriptional silencing (Pannell and Ellis, 2001; Sadelain et al., 2000) and poor levels of tissue-specific expression (Cavazzana-Calvo et al., 2000b) all limit the application of retroviral vectors.

However, as genetic diseases, in particular monogeneic diseases affecting haematopoietic cells, require long-lasting therapeutic gene expression, the potential benefits of the use of gammaretroviral vectors still outweigh the risks, it seems worth exploring methodologies to improve their safety profile without affecting their virtues.

In particular, the many of the problems with gammaretroviral vectors used in gene therapy currently can be traced to their strong transcriptional control sequences within their long terminal repeats (LTRs) and the duplication of these terminal enhancer regions. Together, these characteristics can activate neighbouring genes, either through *cis* acting enhancer interactions or by introducing new promoter activity from their 3'LTR (Baum et al., 2003; Thomas et al., 2003).

Furthermore, other studies have shown positive and negative dosage-dependent effects of transgene expression in mouse and human cells, that the level of expression will likely correlate with risk and in some cases should be kept within a therapeutic level to limit side effects (Baum et al., 2003). Therefore, a reassessment of such risks, vector design, safety testing and the search for possible solutions are at the forefront of the field of gene therapy today (Haviernik and Bunting, 2004; Yi et al., 2005).

The following paragraphs will therefore describe the biology of retroviruses, their modifications as vectors, targeting strategies and their use in gene transfer into haematopoietic stem cells.

1.2.1 Biology of Retroviruses

Retroviruses belong to a large and diverse family of RNA enveloped viruses, the *Retroviridae*, which are united by common features of virion structure and replicative life-cycle. They possess an 8-12 kb RNA genome and the ability to 'reverse transcribe' their genome from RNA to DNA. Commonly, retroviruses contain specific proteins that carry out five basic functions (reviewed by (Coffin et al., 1997)): (1) viral genome condensation into an RNA-protein complex; (2) packaging of this complex in a protein capsid; (3) enclosure of the capsid in a lipid membrane, or envelope; (4) modification of the envelope by displaying surface proteins that recognise cellular receptors; and (5) copying of the retroviral RNA in a newly infected host cell. Although most enveloped viruses are in fact more complex, with either two or more proteins sharing each function, or one protein carrying out several functions, the simpler retroviruses, like murine leukaemia virus (MLV) provide the basic model for the understanding of the structure and functionality of a retrovirus particle (Coffin et al., 1997).

1.2.2 Structure of a retrovirus particle

A retroviral particle (virion) measures about 80-120nm in diameter, which consists of a lipid bilayer envelope derived from the plasma membrane of the host cell with localised retroviral *Env* glycoproteins on its surface (Figure 3). These glycoproteins are formed by two polypeptide subunits: the transmembrane (TM) that anchors the glycoprotein into the envelope lipid bilayer and the surface subunit (SU), which binds to the specific cellular receptors. The virus core, a protein shell formed by capsid proteins (CA), contains two identical copies of plus-strand genomic RNA molecules, which are condensed with nucleocapsid proteins (NC). The retroviral integrase (IN) and reverse transcriptase (RT), which promotes the synthesis of double stranded DNA from the viral RNA template in the host cell, are also contained in the capsid, the inner portion of the virion that defines the viral core (Jones and Morikawa, 1998). Outside the viral core a layer of matrix proteins (MA, membrane associated) interact with the envelope lipid bilayer, which surrounds the viral core particle and localised retroviral protease (PR) molecules.

Figure 3: Schematic structure of a mature retroviral particle

A retroviral virion measures about 80-120nm in diameter and contains two identical copies of the viral RNA genome complexed with nucleocapsid proteins. The structural *gag* proteins consisting of: (MA) Matrix protein, (CA) capsid protein, (NC) nucleocapsid protein; the *Pro-Pol* proteins: (PR) retroviral protease, (IN) integrase and (RT) reverse transcriptase; and the viral (*Env*) envelope glycoproteins: (TM) transmembrane components, (SU) surface components anchored into an envelope lipid bilayer are indicated. Adapted from Retroviruses (Coffin et al., 1997)

1.2.3 Genetic Organisation of the Retroviral Genome

The Genome of all retroviruses is organised into four genes: ***gag, pro, pol*** and ***env*** (Figure 4). The ***gag*** is an acronym for group-specific antigens (ag), and its sequence encodes the three major structural proteins of the viral core comprising: matrix (MA), capsid (CA) and nucleocapsid (NC), the RNA genome binding proteins and the major proteins of the viral core. The *gag* proteins are cleaved by the retroviral protease (PR), which is encoded by the ***pro*** sequence and is responsible for the maturation of viral particles during and after their budding process. The ***pol*** sequence encodes for both, the integrase activity (IN) and the reverse transcriptase (RT) (Baltimore, 1970; Mizutani et al., 1970; Temin and Mizutani, 1970), which carries out the reverse transcription process that takes the retroviral RNA genome to a proviral double-stranded DNA pre-

integration form. The two major functions of the reverse transcriptase during the retroviral life cycle are a DNA polymerase activity and an *RNase*H activity (Moelling et al., 1971) that functions as a ribonuclease during the reverse transcription of the viral genome by degrading the RNA template from RNA-DNA hybrids prior to its integration into the host genome. The integrase (IN) is a separate protein derived from the carboxy terminus of the *pol* gene, which catalyses the integration of the proviral DNA into the host genome. The ***env*** sequence encodes a polyprotein that is post-translationally cleaved into two subunits of the envelope glycoproteins: the transmembrane domain (TM) and an external glycosylated surface domain (SU) which determines the host range of the virus.

Figure 4: Genetic organisation of a generalised provirus

The top double line shows the proviral DNA as it is inserted into the host DNA sequence, flanked at each end with the long terminal repeats (LTRs) composed of U3, R, and U5 elements. LTR sequences that are important for transcription, e.g. the enhancer, promoter, and the poly(A) addition signal, are indicated. The *gag*, *pro*, *pol*, and *env* sequences are located conserved in the positions shown in all retroviruses. Accessory genes are located as indicated, which can also overlap *env* and U3 and each other, and occasionally in other locations. The second line shows the RNA that is the primary transcriptional product. Important sequences for replication and gene expression are marked at the approximate positions in which they are typically found, e.g. (PBS) Primer-binding site; (ψ) psi packaging signal sequence; (SD) splice donor site; (FS) frameshift site; (SA) splice acceptor site; (PPT) polypurine tract; (PA) polyadenylation signal; and (AAA) poly(A) tail. The third line, at the bottom, indicates the spliced messenger RNA for the *Env* protein. Retroviruses with accessory genes have other spliced mRNAs, and contain other splice donor and splice acceptor sites as well. Adapted from Retroviruses (Coffin et al., 1997).

In addition more 'complex' retrovirus, like lentiviruses have additional accessory genes, which regulate viral gene expression, assembly of infectious particles and modulate viral replication in infected cells (Tang et al., 1999).

1.2.4 Long terminal repeats (LTRs)

In simple retroviruses such as the Moloney murine leukemia virus (Mo-MLV), the LTR sequences contain most *cis*-acting elements required for the transcriptional initiation and expression of the viral genes as described above. The retroviral cap site is defined by the 5`end of the R region. The R region forms a direct repeat at both ends of the LTR and provides the homology sequence necessary for strand transfer during the reverse transcription process in which the U5 and U3 regions are duplicated. The 3`R is polyadenylated with about 50-200 noncoding adenylate residues(Coffin et al., 1997; Palu et al., 2000).

Transcription is regulated by the sequences within the U3 region of the LTR, which can be distinguished into the promoter region with TATA box and a set of positive *cis*-acting regulatory signals that lie immediately upstream (Graves et al., 1986) and the enhancer region approximately 150bp upstream of the TATA element, which constitutes two 75bp direct repeats (Levinson et al., 1982) that control gene expression and tropism in different cellular environments through a variety of cellular transcription factors binding sites, which are closely packed or overlapping (Figure 20)(Coffin et al., 1997; Palu et al., 2000) .

1.2.5 Reverse Transcription of the viral RNA genome

The process of reverse transcription of the viral RNA genome yields in a linear DNA double stand by which the U5 and U3 regions are duplicated (Figure 5). As a result the proviral DNA product is longer than the RNA template at both ends, which is the origin of the two long terminal repeats (LTRs), each consisting of U3, R and U5 regions that are characteristic to the proviral DNA form of the retroviral genome.

Figure 5: Reverse transcription of viral RNA to Viral DNA (provirus)

The integrated provirus is larger than the viral genome but its complexity is the same. Due to the duplication of U3 and U5 during reverse transcription, the wild-type provirus is able to initiate a new round of replication. Adapted from Retroviruses (Coffin et al., 1997).

Reverse transcription is initiated by the reverse transcriptase by complementary alignment of the 3`terminus of a primer t-RNA to the primer binding site (PBS), which is located partly within the untranslated region (leader region) at the 5`end of the retroviral genome, next to the 3`end of the U5 region. The step-by-step process of reverse transcription is described and represented in Figure 6.

Figure 6: Step-by-step Process of Reverse Transcription

The location of the R, U5, and U3 regions, the polypurine tract (PPT), and the primer-binding site (PBS) are indicated. The entire process is catalyzed by reverse transcriptase which has both DNA polymerase and RNase H activities. (black coloured line) RNA; (orange coloured line) minus-strand DNAs; (red coloured line) plus-strand DNA. (1) A retrovirus-specific cellular tRNA hybridizes with a complementary region called the primer-binding site (PBS). (2) A minus-stand DNA segment is extended from tRNA based on the sequence of the retroviral genomic RNA. (3) The viral 5`R and U5 sequences are removed by RNase H. (4) First jump: DNA hybridises with the remaining R sequence at the 3`end. (5) The minus DNA strand is extended from the 3`end. (6) Most viral RNA is removed by RNase H, the polypurine tract (PPT), located upstream of the U3 functions as the initiation site of positive strand DNA synthesis. (7) The second (positive) DNA strand is extended from the viral RNAs polypurine tract (PPT), and both tRNA and the remaining viral RNA are removed by RNase H. (8) Second jump: The PBS region of the second strand hybridises with the PBS region of the first strand. (9) Extension on both DNA strands by DNA synthesis completing the provirus flanked by LTRs. Adapted from Retroviruses (Coffin et al., 1997).

1.2.6 The Retroviral Life cycle

The life cycle of a replication-competent retrovirus begins in the nucleus of an infected cell and is illustrated in (Figure 7). At this stage the retroviral genome is a double-stranded DNA element integrated into and covalent attached to the DNA of the host genome. The transcription of full-length retroviral RNAs is initiated at the beginning of the 5`LTR. The viral RNA transcripts undergo splicing and polyadenylation events in the cell nucleus of the host cell, similar to those of the host cell RNA transcripts and are translocated to the cytoplasm by nuclear export. Unspliced full-length viral RNA molecules act as template for the translation of the *gag-pol* proteins, while spliced RNA species lead to the production of envelope proteins. Gag and pol are translated as either gag protein, or a *gag-pol* polyprotein precursor. The viral protease cleaves the precursor proteins into multiple subunits. Envelope glycoproteins are also translated as a precursor, which are then cleaved by endogenous proteases to yield the mature surface glycoproteins. The cleaved proteins translocate at the cell surface and assemble to retroviral core particles, which encapsidate two molecules of full-length unspliced genomic viral RNAs. Hereby the genomic viral RNA species are identified and distinguished from other RNA in cells by a special psi sequence (packaging signal, ψ) (Joshi et al., 1990), which is required for encapsidation of the RNA into newly formed virion particles that are released by budding and proteolytic maturation from the cell.

Figure 7: A schematic representation of the life cycle of a retrovirus

The life cycle of a retrovirus begins with the (1) transcription of the proviral DNA genome into full-length RNA transcripts. (2) RNA transcripts undergo splicing events in the nucleus of the host cell. (3) Nuclear export of spliced RNA species. (4) Translation of the RNA genome into viral proteins and (5) packaging and assembly of genomic RNA into viral core proteins. (6) release of new progeny by budding and proteolytic maturing of virus particles. (7) Adsorption to specific receptor to a new host cell. (8) Fusion of the membranes with loss of envelope and (9) entry of the viral core into the cytoplasma of the host cell. (10) reverse transcription of the viral RNA template to double stand DNA. (11) Nuclear translocation of retroviral DNA into the nucleus and integration into the host genome until a new round of replication is initiated. Adapted from Retroviruses (Coffin et al., 1997) and (Palu et al., 2000).

The free virus particle can infect new cells by binding to specific cell surface receptors. The infection process begins with the interaction of the envelope proteins of the viral particle with the plasma membrane of the target cell, which primarily determines the specificity of the virus–cell interaction. Binding of the viral glycoprotein to a specific receptor complex on the cell surface causes viral and cell membranes to fuse, which process leads to injection and uncoating (loss of envelope) of the virus core particle into the cell cytoplasma. Once inside the cell, the viral core is partially degraded to form a large nucleoprotein particle containing the viral genome (preintegration complex).

During this process, the retroviral reverse transcriptase reverse transcribes the viral RNA genome into double-stranded DNA copies of the proviral genome as shown in (Figure 5). Upon completion of reverse transcription, the viral integrase, mediates integration of the double stranded proviral DNA into the host chromosome.

The process of entry into the nucleus differs between gamma-retroviruses (e.g. MLV) and lentiviruses (e.g. HIV). For lentiviruses it has been shown that efficient transport to the nucleus is active by utilisation of the host cells nuclear import machinery (Bukrinsky et al., 1993), while the entry of MLV transcripts to the nucleus are dependent on mitosis when the nuclear membrane is disassembled, but can not transit through the pores of the nuclear membrane of quiescent cells (Lewis and Emerman, 1994; Roe et al., 1993).

As provirus, the retroviral DNA is fully integrated as part of the host genome until the provirus is transcribed to give rise to new viral genomic RNA and viral proteins required for the formation of new virions, thereby initiating a new round of replication and complete a new retroviral life cycle (Palu et al., 2000).

1.2.7 Development of recombinant retroviral vectors

As retroviruses are potentially pathogenic, their clinical use implies the development of vectors carrying the therapeutic sequence(s), but are replication-incompetent and are unable to transfer viral functions. Hence, the retroviral genome was trimmed down to provide in *cis* just the signals essential for replication and packaging of vector RNA, while packaging cell lines were developed to express and supply the viral genes *gag*, *pro*,

pol and *env* in *trans* using molecular constructs which can not be packaged into retroviral particles.

1.2.8 Generation of recombinant Retroviral Vectors

As described above, replication-competent retroviruses (such as wild-type retroviruses) carry *gag*, *pol* and *env* genes. Their genome also bears a *cis*-acting sequence (ψ) necessary for encapsidation into viral capsids.

By contrast retroviral vectors lack *gag*, *pol* and *env* which are replaced by another gene (for example, a therapeutic gene) which renders them replication incompetent. Vectors therefore are transfected into 'packaging cells' that constitutively express *gag*, *pol* and *env* genes from transfected expression plasmids, to produce recombinant retroviral vectors. The RNA that is transcribed from the retroviral vector carries no *gag*, *pol* or *env* sequences, but only the gene(s) of interest. It can be packaged into viral capsids, owing to the inclusion of the encapsidation sequence (Ψ). Retroviral vectors produced in such a manner can infect other permissive cells, but they can no longer replicate because they do not carry *gag*, *pol* and *env* genes.

Since recombinant retroviral vectors for gene delivery must be replication-incompetent their genome was trimmed down to provide in *cis* just the signals essential for replication and packaging of vector RNA. The remaining non-essential signals and packaging components are provided *in trans* by the helper system in producer cells. Such helper-free packaging lines are capable of producing all the necessary *trans* proteins - *gag*, *pol*, and *env* - that are required for packaging, processing, reverse transcription, and integration of recombinant genomes. In this configuration the capacity of the vector to accommodate exogenous DNA/RNA without losing its packaging capacity is restricted to the size of an insert of maximally ~7-7.5 kb (Verma and Somia, 1997).

Retroviral vectors produced in such a manner can infect other permissive cells, but they can no longer replicate, because they do not carry the *pol* and *env* genes.

Figure 8: Schematic structure of a packaging cell.

The packaging functions (*gag*, *pro*, *pol* and *env*) are supplied in *trans* using separate molecular constructs. Only the recombinant retroviral vector expressing the therapeutic gene is packaged into newly formed virions without replication function. Adapted from (Marin et al., 1997)

The production of recombinant vectors is induced by transfection of the recombinant vector into 'packaging cells' that constitutively express *gag*, *pol* and *env* genes. For safety reasons by the production of recombinant retroviral vectors, the *gag*, *pol* and *env* gene are replaced by the gene of interest, e.g. a reporter gene and/or the therapeutic gene.

Recombinant retroviruses were first pioneered by Richard Mulligan and David Baltimore with the psi-2 lines and analogous retrovirus packaging systems, based on NIH 3T3 cells (Mann et al., 1983).

The three most commonly used packaging cell lines are: the amphotopic PA317 (Miller and Buttimore, 1986), the amphotrophic GP+envAM12 (Markowitz et al., 1988), and the gibbon ape leukaemia virus based PG13 (Miller et al., 1991). Later, a human fibrosarcoma cell line HT1080 was selected for their ability to release high-titre viruses that are resistant to inactivation by human serum. Two HT1080-based packaging cell lines were created, which produce Moloney murine leukemia virus cores with envelope glycoproteins of either amphotropic murine leukemia virus (FLYA13 line) or cat endogenous virus RD114 (FLYRD18 line) origin (Cosset et al., 1995). However, due to lengthy process of identifying a high-titre producer cell clone, transient packaging systems, like the 3 plasmid packaging system with COS and/or 293T/17 cells demonstrated to be the system of choice, where rapid high-titre and helper free viral production is required (Soneoka et al., 1995). Second-generation retrovirus producer lines for the generation of helper free ecotropic and amphotropic retroviruses called Phoenix™ by (Kinsella and Nolan, 1996) were soon developed. The latter two packaging systems will be described in more details later in this thesis.

1.2.9 Retroviral mediated gene therapy for CGD

As an attractive candidate disorder for correction by gene therapy, chronic granulomatous disease (CGD) has been used as a model system by a number of groups in the field looking to develop improved therapies to those currently available (Barese et al., 2004; Curnutte, 1993; Curnutte and Babior, 1987; Dinauer et al., 2000; Dinauer, 2005; Goebel and Dinauer, 2003; Goldblatt and Thrasher, 2000; Grez et al., 2000; Kume and Dinauer, 2000; Malech et al., 1997b; Malech et al., 1997a; Malech, 1999; Ott et al., 2006; Porter et al., 1993; Porter et al., 1996a; Schwickerath et al., 2004; Stein et al., 2006; Thrasher et al., 1992; Thrasher et al., 1993; Thrasher et al., 1994; Thrasher et al., 1995; Winkelstein et al., 2000).

The first successful *in vitro* correction of CGD was achieved by retroviral mediated gene transfer into B cell lines derived from $p47^{phox}$-deficient patients (Thrasher et al., 1992). Similar correction of X-CGD cell lines followed soon after (Porter et al., 1993). *In vitro* correction of superoxide generation was subsequently achieved in colonies derived from

transduced CD34+ cells harvested from CGD-patient peripheral blood firstly, for $p47^{phox}$-deficient (Sekhsaria and Malech, 1993) and subsequently for both forms of cytochrome b_{558}-deficient (Li et al., 1994) and the remaining autosomal recessive forms of CGD (De Leo et al., 1996; Porter et al., 1996b).

High levels of correction of bone marrow-derived long term culture initiating cells (LTC-ic) from X-CGD patients were also reported (Porter et al., 1996a). On the basis of these results a clinical trial of CGD gene therapy was instigated at NIH (Barese et al., 2004; Malech et al., 1997b). Although the levels of superoxide generation and corrected circulating neutrophils were vanishingly low, ranging between 0.004% and 0.6%, sustained transgene expression was achieved, without any untoward effects on the patients' health (Malech et al., 1997b; Malech et al., 1997a). These levels of phenotypic correction were clearly still some orders of magnitude below therapeutically useful levels, suggesting that more work was required to improve gene expression and the overall efficiency of transduction as gene corrected myeloid cells do not have any proliferative selective advantage in this condition.

More recently, with the advances in bone marrow pre-conditioning, a clinical trial for CGD reported the achievement of significant clinical benefits to chronic infections for two patients (Ott et al., 2006; Ott et al., 2007). Impressive levels of corrected neutrophils (10-30%) in peripheral blood were found in the first few month post-transplant, which rapidly increased to 50-80% after 4-6 month. It was found that most of the corrected cells originated from the clonal expansion of myeloid precursors were the vector integration had occurred in or close to the transcriptional start site of three growth promoting genes, which led to abnormal haematopoiesis.

All the retroviral vectors used in the studies outlined above made use of the endogenous retroviral long terminal repeat (LTR) to control expression of the therapeutic gene.

The LTR acts as a constitutive promoter/enhancer in many different cells and tissue types, whilst certain strains of retroviruses show preferential transcriptional tropism, they are also known to contain sequences that result in the suppression of efficient expression of genes from the viral enhancer. This is especially true for vectors transduced into embryonic stem cells, where the phenomenon of silencing *in vivo*, in

which output from the gene gradually reduces with time, is well established (Grez et al., 1990; Pannell and Ellis, 2001; Robbins et al., 1997; Robbins et al., 1998; Soriano et al., 1991).

One strategy to improve the efficiency of gene therapy for CGD, has been to try to construct retroviral vectors capable of high-level, sustained expression specifically in myeloid cells. This might be achievable by substituting the viral promoter/enhancer elements of the LTR with those derived from genes with promoters that confer high level myeloid-cell-specific transcription patterns.

The molecular basis of myeloid-cell-specific transcription is therefore described below.

1.3 Myeloid specific Gene Expression

The term *myeloid* is derived from the Greek word for marrow, and myeloid cells constitute the main cellular population of the bone marrow, namely granulocytes, monocytes and their committed progenitors. Myeloid cells arise from pluripotent haematopoietic stem cells (HSCs), which represent a rare population in the bone marrow – estimated to be approximately one per 10^5 marrow cells (Rosmarin et al., 2005). The ability of genetic manipulation of HSCs, controlling and directing expression of a transduced therapeutic gene to mature blood cells lineages in a tissue- or cell-specific manner has been of particular interest to gene therapy applications for inherited monogenetic blood disorders and requires an understanding of haematopoiesis.

1.3.1 Haematopoiesis

Haematopoiesis is defined as the life-long process of pluripotent HSC proliferation, self-renewal and differentiation that is responsible for the production of mature blood cells (Keller, 1992). The process is driven by complex coordinated patterns of gene expression under the influence of cytokines and hormones whereby pluripotent HSCs undergo successive symmetric and asymmetric divisions involving self-renewal, differentiation and maturation to yield committed progenitor cells of distinct blood cell

lineages (lymphoid and myeloid) (Dexter and Heyworth, 1994). A schematic model of haematopoiesis is presented in Figure 9.

Committed lymphoid progenitor cells give rise to T-cells and B-cells as well as natural killer (NK) cells. In the lymphocyte lineage, B- and T-cells represent 25-35% of all leukocytes that take part in antibody mediated immunity and cell mediated immunity. B-cells circulate the blood and lymphatic systems, and can mature into plasma cells that secrete antibodies. T-cell precursor undergo differentiation in the thymus into two distinct types of T-cells, T-helper (Th) cells, which are CD4+, and the cytotoxic T (Tc) cells, which are CD8+.

Myeloid progenitor cells give rise the red blood cells: erythrocytes, the cells that carry oxygen and megakaryocytes, the precursor of blood platelets that facilitate blood clotting. In particular, the myeloid lineage gives rise to the white blood cells, the second group of leukocytes that provide innate immunity and subdivided into: neutrophils, eosinophils, basophils, and monocytes. The latter, which replenish resident marcophages and dendritic cells.

The majority of cells of the myeloid lineage are granulocytes, especially neutrophils, which at 50-70% of all leukocytes represent the largest group of nucleated cells in the blood, and macrophages; these two cell types being those most active in phagocytosis (as described in 1.1.1). Eosinophils represent with about 5% of leukocytes and are important in the elimination of pathogens too large to be engulfed. Mast cells are fixed in tissues and are thought to originate from the same precursor cell as circulating basophils (1% of all leukocytes), together they play a key role in the inflammatory process.

Macrophages and dendritic cells are derived from monocytes (3-9%) and mediate, as one of several bridges, between innate immunity and adaptive immunity, by presenting antigens to immunocompetent T-cells.

Figure 9: Schematic model of haematopoiesis.

Pluripotent haematopoietic stem cells (HSCs) derived from the bone marrow have the ability to self-renew and to differentiate into a variety of lineages as indicated. Haematopoietic stem cells can become committed lymphoid or myeloid precursors (common lymphoid and myeloid progenitors, respectively). The lymphoid lineage produces B cells, T cells and natural killer (NK) cells. The myeloid lineage produces erythrocytes (red blood cells), megakaryocytes (precursor of blood platelets), mast cells, basophils, eosinophils, neutrophils, and monocytes. Adapted from (Qasim et al., 2004))

1.3.2 Myeloid specific promoters, genes and transcription factors

Distinct lineages are characterised by the expression of lineage-specific genes. Such genes are regulated by the binding of a unique combination of transcription factors to recurring promoter motifs.

1.3.2.1 Myeloid specific promoters and genes

The proximal regions of myeloid cell-restricted genes often display lineage-restricted activity in transient transfection assays. Commonly, myeloid-specific promoters lack TATA boxes, but have a clearly defined site of transcription initiation.

These include the promoters of the genes that encode the leukocyte integrin subunit CD11b (Pahl et al., 1992), immunoglobulin receptor FcγR1 (CD64) (Eichbaum et al., 1994; Perez et al., 1993), immunoglobulin receptor FcγRIII (CD16) (Feinman et al., 1994), macrophage colony-stimulating factor (M-CSF) receptor (Zhang et al., 1994a; Zhang et al., 1994b), granulocyte colony-stimulating factor (G-CSF) receptor (Smith et al., 1996a), granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor (Hohaus et al., 1995), transcription factor PU.1 (Chen et al., 1995a), endotoxin lipopolysaccharide receptor CD14 (Zhang et al., 1994c), proto-oncogene c-fps/fes (c-fes) (Heydemann et al., 1996), macrophage scavenger receptor (Moulton et al., 1994), prointerleukin 1beta (IL-1β) (Kominato et al., 1995), macrophage inflammatory protein (MIP)1α (Grove and Plumb, 1993), neutrophil elastase (Oelgeschlager et al., 1996; Srikanth and Rado, 1994), CD11c integrin gene (Noti et al., 1996), leukocyte integin CD18 (Rosmarin et al., 1995), leukocyte integin CD11d (Noti et al., 2000), phagocyte $p47^{phox}$ (Li et al., 1997), haematopoietic cell kinase (HCK) (Hauses et al., 1998), interleukin-8 (IL-8) receptor (CXCR1) (Wilkinson and Navarro, 1999), cell surface glycoprotein CD36 (Armesilla et al., 1996), transmembrane ectopeptidase gene of aminopeptidase N (APN)/CD13 (Shapiro et al., 1991), proteinase 3 (Sturrock et al., 1996), neutrophil peptide defensins (Tsutsumi-Ishii et al., 2000), and lactoferrin (Khanna-Gupta et al., 2000).

In the position at which TATA-boxes are typically located, binding sites for the *Ets* family transcription factor PU.1 are repeatedly present instead, which are employed to determine the transcriptional start site for these genes, and PU.1 has been shown to interact with TATA-binding protein directly (Hagemeier et al., 1993).

PU.1 plays a key role in myeloid development and myeloid-specific gene regulation. It has been demonstrated to be truly tissue-specific for myeloid cells and B cells but is not present in T cells and HeLa (non-haematopoietic) cells (Chen et al., 1995a; Chen et al.,

1995b). In particular, PU.1 has been shown to be essential for $p47^{phox}$ promoter activity in myeloid cells (Li et al., 1997; Marden et al., 2003b). It has also been shown that PU.1 regulates the expression of the human neutrophil elastase gene (Srikanth and Rado, 1998) and that it is the essential activator for the expression of $gp91^{phox}$ gene in human peripheral neutrophils, monocytes, and B lymphocytes (Suzuki *et al.*, 1998). PU.1 (Spi-1) regulates the myeloid-cell-specific *c-fes* promoter (Heydemann et al., 1996) and plays a critical role in the tissue-specific expression of cell surface marker CD14 in monocytic cells (Zhang et al., 1994c).

In contrast, the inhibition of PU.1 function in human CD34+ progenitors has been shown to block myeloid colony formation. Targeted mutagenesis of the murine PU.1 locus has revealed the fundamental role this protein plays in blood cell differentiation at all stages of haematopoiesis (yolk sac, fetal liver and bone marrow). For example, PU.1 has been disrupted by two independent research groups and both strains of PU.1-deficient mice exhibit abnormalities in B cell, T cell, monocyte and neutrophil development (Simon, 1998). Other gene disruption studies have shown that the expression of PU.1 is not essential for early myeloid lineage or neutrophil commitment in mice, but is essential for monocyte/macrophage development (Anderson et al., 1998a; Anderson et al., 1998b).

1.3.2.2 Transcription factors involved in myeloid-specific gene expression

Transcription factors play a vitally important role during myeloid differentiation. However, there is no single master myeloid transcription factor that governs myeloid linage commitment alone (Rosmarin et al., 2005). Instead, multiple transcription factors work cooperatively and coordinately to regulate both temporal and linage-specific gene expression (Shivdasani and Orkin, 1996). More than a dozen transcription factors are pivotal to the regulation of myeloid genes (Friedman, 2002; Skalnik, 2002; Tenen, 2003; Zhu and Emerson, 2002).

Transcriptions factors for myeloid-specific gene expression have been divided into several families: the CCAAT/enhancer binding protein (C/EBP) family, with C/EBPα and C/EBPβ; the Runt/PEBP2/CBF family including AML1/PEBP2αB/CBFα2 and

the *Ets* family, with PU.1 and Ets-1 or Ets-2, the ubiquitous AP-1 group and SP1 a single, universally expressed protein, associated with promoters without TATA boxes (Dynan et al., 1985).

In particular, PU.1 and the CCAAT/enhancer binding protein C/EBPα are specifically required for normal development and differentiation of the myeloid linage by regulating several myeloid specific genes. However, neither PU.1 nor C/EBPα is exclusively myeloid restricted, while PU.1 is also expressed by B lymphocytes and C/EBPα is expressed by liver and other organs.

In the haematopoietic system C/EBPα has only been found in the myeloid lineage and is thought to be important in early commitment of progenitor cells because the factor is down-regulated when myelomonocytic cells are induced to differentiate (Natsuka et al., 1992; Scott et al., 1992). C/EBPα binding sites are critical for myeloid-specific expression of neutrophil elastase (Oelgeschlager et al., 1996) and the receptors for macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF) (Hohaus et al., 1995; Smith et al., 1996a; Zhang et al., 1996).

Essentially, myeloid cell-restricted gene expression is due in part to the combinatorial action of multiple transcription factors present in overlapping sets of haematopoietic cells.

For example, PU.1 and Sp1 activate c-fes, CD11b, and CD18 gene promoters (Chen et al., 1993); PU.1 cooperates with CCAAT/enhancer-binding protein-α (C/EBPα), c-myb, and AML1 in the activation of the neutrophil elastase gene promoter (Nuchprayoon et al., 1994; Nuchprayoon et al., 1997; Oelgeschlager et al., 1996); PU.1, AML1, and C/EBP activate the M-CSF receptor promoter (Zhang et al., 1994a; Zhang et al., 1996); and PU.1 cooperates with C/EBPβ in the activation of the IL-1β promoter (Yang et al., 2000), and with C/EBPα to activate the G-CSF receptor promoter (Smith et al., 1996a). In addition, AML1 and c-myb acting together in the activation of the myeloperoxidase gene promoter (Britos-Bray and Friedman, 1997), and C/EBP and Sp1 cooperate in the activation of the CD11c promoter (Lopez-Rodriguez et al., 1997).

In contrast to the results of transient transfection experiments, distant *cis* regulatory elements are often required in transgene constructs to achieve myeloid specific gene expression *in vivo*, although in most cases a detailed understanding of the mechanisms responsible for directing appropriate myeloid cell-restricted expression are unknown (Skalnik, 2002).

1.3.3 A 'minimal' myeloid-specific promoter

Studies of myeloid-specific promoter function in transgenic mice indicated that fully regulated expression requires extensive regulatory sequence. For many myeloid-specific genes, however, a relatively short 5' proximal promoter (generally 300bp or less) is capable of directing myeloid-specific expression *in vitro*.

For example, 128bp of the CD14 promoter can direct macrophage-specific expression (Zhang et al., 1994c); 70bp of the GM-CSFαR promoter directs myeloid-specific expression (Hohaus et al., 1995); 106bp of the neutrophil elastase sequence directs myeloid-specificity (Srikanth and Rado, 1994); 74bp of the G-CSFR promoter directs myeloid-specific expression (Smith et al., 1996a); the majority of the myeloid-specificity and activity of the M-CSFR promoter is contained within 85bp (Zhang et al., 1994a); 412bp of the CD11b promoter directs myeloid-specific expression, with the proximal 92bp retaining significant myeloid expression (Chen et al., 1993; Pahl et al., 1992); and 446bp of the c-*fes* promoter was found to direct myeloid-specific expression in transgenic mice (Heydemann et al., 2000).

A comparison of 'minimal' promoter sequences highlights a number of common features. *Firstly*, a core promoter sequence commonly required for initiating basal transcription (TATA or *Inr* sequence) is often absent. *Secondly*, a functional binding site for the *Ets* family transcription factor PU.1 is located close to the transcriptional start site. *Thirdly*, a binding site for at least one other transcription factor is located in close proximity to the PU.1 binding site. Any additional factors are generally of a restricted group that includes the CCAAT/enhancer-binding protein-α (C/EBPα), polyoma virus enhancer binding factor 2/core binding factor (PEBP2/CBF) and the ubiquitous factor Sp1.

While evidence that PU.1, Sp1 and C/EBPα can interact with the TATA-binding protein component of the general transcription factor TFIID *in vitro* (Emili et al., 1994; Hagemeier et al., 1993; Nerlov and Ziff, 1995) suggests that in the absence of classical core promoter sequences, proximal binding factors may facilitate recruitment of the basal transcriptional apparatus.

1.4 Objectives and aims of this investigation

As part of a strategy to develop gene therapy for chronic granulomatous disorder (CGD), this project aimed to improve the transcriptional output of retroviral vectors in myeloid cells, the cell type affected in CGD.

As indicated above, successful retroviral vectors have commonly made use of the endogenous retroviral long terminal repeat (LTR) for expression of inserted genes. The LTR is a potent constitutive promoter/enhancer that functions well in many different cells and tissue types, but has been associated with insertional mutagenesis, dysregulation, transgene silencing, and lack of specificity.

Our approach to improving the levels of myeloid cell gene expression from a retroviral vector was to take advantage of cellular promoters from endogenous genes that naturally have myeloid-cell-specific transcription patterns, and to construct vectors that substitute the viral enhancer elements with those derived from the cellular promoters. In this way we hoped to optimise expression from the transgene in successfully transduced myeloid cells.

As an additional benefit it was surmised that this may also help to improve the safety profile of integrating vectors like retroviruses, by reducing the likelihood of downstream promotion, or localised viral enhancer-mediated up-regulation of adjacent genes.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents and Buffers

Unless otherwise stated, all reagents were obtained from Sigma. In some cases the cat.# is given, if the exact supplier and batch is essential. Radioactive isotopes [γ^{32}P]-ATP, and [^{35}S] dATP were obtained from ICN Biomedical. Distilled MilliQ water was used to prepare all solutions, which were autoclaved at 121°C for 20 min to sterilise.

Reagents and buffers used are listed as follows:

2.1.1.1 General Reagents and Buffers

50× Tris acetate buffer (TAE): 0.2M Tris base, 1M glacial acetic acid (BDH), 50mM EDTA, pH8.0.

6× DNA loading buffer: 30% Glycerol (v/v), 20mM EDTA, 0.2% bromophenol blue (w/v).

PBS: PBS tablets (Oxoid) were dissolved 1 per 100 ml water and autoclaved.

Sephadex: Sephadex G50 was dissolved in distilled water, washed 2-3 times and resuspended in TEN at 10% (w/v).

TE: 10mM Tris-HCl, pH8.0, 1mM EDTA

TEN: 100mM NaCl, 10 mM Tris-HCl, pH8.0, 1mM EDTA, pH8.0

Tris borate buffer (TBE): 90mM Tris-HCl, 90mM boric acid, 1 mM EDTA, pH8.0.

T4 Ligase 10× Buffer (Promega): 300mM Tris-HCl (pH7.8), 100mM $MgCl_2$, 100mM DTT and 10mM ATP. Aliquots were stored at –20°C.

2.1.1.2 Plasmid extraction solutions

Cell Suspension solution (Spin Kit or Flow Kit, HYBAID, UK): 50mM Tris-HCl (pH8.0), 10mM EDTA, 100μg/ml RNase A

Cell Lysis Solution (Spin Kit or Flow Kit, HYBAID, UK): 200mM NaOH, 1% SDS (w/v)

Neutralising solution (Spin Kit, HYBAID, UK): 3.2M potassium acetate/acetic acid (pH5.5) and guanidine hydrochloride, which is essentially different to the HYBAID Flow Kit

Wash Solution (Spin Kit, HYBAID, UK): contains ethanol, NaCl, EDTA and Tris-HCl

Resuspension Solution (Flow Kit or Spin Kit, HYBAID, UK): 50mM Tris-HCl (pH8.0), 10 mM EDTA, 100μg/ml RNase A

Neutralising Solution (Flow Kit, HYBAID, UK): 3.2M potassium acetate/acetic acid (pH5.5) only, which is essentially different to the HYBAID Spin Kit

Equilibration Solution (Flow Kit, HYBAID, UK): 600mM NaCl, 100mM sodium acetate/ acetic acid (pH5.0), 0.15% Triton X-100

Wash Solution (Flow Kit, HYBAID, UK): 800mM NaCl, 100 mM sodium acetate / acetic acid (pH5.0)

Elution Solution (Flow Kit, HYBAID, UK): 1250mM NaCl, 100mM Tris-HCl (pH8.5)

2.1.1.3 Prokaryotic culture medium

LB (Luria-Bertani) bacterial growth medium: 2% LB broth base (w/v) (GibcoBRL), solid media was supplemented with 15% bacto-agar (w/v) (Difco). All media were allowed to cool below 50°C before addition of Ampicillin, 100mg/ml or Kanamycin, 40mg/ml, respectively.

2.1.1.4 Eukaryotic culture medium

RPMI medium: RPMI 1640 (GibcoBRL), 10% FCS (v/v), 2mM L-Glutamine (GibcoBRL), 10μg/ml penicillin/streptomycin.

Freezing Medium: 90% heat-inactivated fetal calf serum (FCS), 10% DMSO (v/v)

Growth Medium (GM) for 293T Cells and Derivatives. The following are added directly to DMEM (Dulbecco's MEM with Glutamax (GibcoBRL) to give the indicated final concentrations: 10% heat inactivated fetal calf serum, 100U/ml Penicillin, 100U/ml Streptomycin, 2mM L-glutamine

Hygromycin B (Boehringer Mannheim, cat.# 843-555 or Sigma, cat.#H3274): 250× stock solution (1g/50ml) was applied at 250-500μg/ml final concentration.

Diphtheria toxin (Calbiochem, cat.#322326 or Sigma, cat.#D2918): 1g diphtheria toxin from *Corynebacterium diphtheriae* dissolved in sterile PBS with 0.1% BSA, aliquots stored at –80°C, used at 1-2μg/ml on cells, select 1 week

PMA: phorbol myristate acetate (Sigma), 1ng/ml

Polybrene: 4mg/ml (dissolved in PBS and subsequently filtered through a 0.2 μm filter and stored at either 4°C or –20°C) is the stock concentration, used at 4-8μg/ml during infection on cells.

Flow cytometry staining buffer: PBS, 1% BSA (w/v), 0.01% sodium azide (w/v).

2.1.1.5 Calcium Phosphate Coprecipitation Transfection Buffers

1× HEPES: 21 mM HEPES, 0.137M NaCl, 5mM KCL, 0.7 mM Na_2HPO_4, 5.5 mM glucose, pH7.1

2x HBS: 5.25g Na_2HPO_4 dibasic (FW 141.96, BDH) dissolved in 500ml of water for stock solution. 2× HBS was made of 8.0g NaCl, 6.5g HEPES (sodium salt, from Sigma cat.#H-7006) and 10ml Na_2HPO_4 stock solution. Because the pH7.05±0.05 is very important, it is recommended to make 3 batches pH6.95, pH7.0 and pH7.05 using NaOH or HCl, filled up to a total volume of 500ml and tested for which one yields the best precipitate.

$CaCl_2$ solution: 2.5M $CaCl_2$ solution was filtered through a 0.2 μm filter, aliquoted and stored at –20°C.

Chloroquine: 25 mM chloroquine stock solution is prepared in neither PBS or growth medium (GM) and sterile filtered through a 0.2 μm filter and stored at –20°C.

2.1.1.6 Northern Blot buffers

Hybridisation solution: 5× Denhardts (v/v), 100μg/ml sonicated salmon sperm DNA (denatured at 98°C for 5 min), 5× SSC (v/v), 0.5% SDS (w/v).

Denaturing solution: 1.5M NaCl, 0.5M NaOH.

DEPC water: 0.1% DEPC (v/v), incubated overnight at room temperature (RT) and autoclaved.

20×SSC: 3M NaCl, 0.3M sodium citrate

2.1.1.7 Western Blot buffers

Lysis buffer (Western): 4100μl water, 500μl 10% NP40, 100μl 1M Tris pH8.0, 130μl 5M NaCl, 100 μl 500mM NaF, 50μl mM PMSF, 10μl Na_3VO_4, combined in a bjiou-tube, sterile filtered and store at 4°C. 1μl 5000× leupeptin, 5000× pepstatin and 5 μl 1M DTT was added to this lysis buffer just before use.

Main gel buffer (Western): dissolve 91.05g Tris in 400ml water, adjust pH8.8 with HCl, add 2.5ml of 20% SDS solution and filled up to a total volume of 500ml with water

2× SDS buffer (Western): dissolve 0.76g Tris and 0.5mg bromphenol blue in 5ml 20% SDS, 10 ml glycerol and 20 ml water. Adjust pH to 6.8 with HCl and fill up to 50ml and store at RT.

SDS-PAGE Running buffer (Western): 3g Tris, 14.4g glycine filled to 997.5ml with water. Add 2.5ml 20% SDS and mix. Alternatively, prepare a 5× stock with 15g Tris, 72g glycine and 12.5ml 20% SDS filled to 1l with water, mix and bottle. 200ml of 5× stock can be diluted with 800ml water to yield a 1× buffer

2× SDS loading buffer: 125mM Tris-HCl, pH6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 2% 2-mercaptoethanol, 0.001% (w/v) bromophenol blue (BDH).

Stacking buffer (Western): 30.35g Tris dissolved in 400ml water, pH to 6.8 with HCl. Add 10ml of 20% SDS and top-up to 500ml with water, mix and bottle

Stripping buffer (Western): combine 347µl β-mercaptoethanol, 12ml Tris-HCl pH6.7, 5ml 20% SDS, 41ml water

Transfer buffer (Western): 10ml 10× transfer buffer (58.2g Tris and 29.3g glycerine dissolved in 900ml water, pH to 9.2 and fill up to 1l, mix and bottle) with 70ml water and 20ml methanol, mix and use immediately.

SDS buffer (RNA preparation): 3.8% SDS (w/v), 0.038M EDTA pH8.0, 3.8 mg/ml proteinase K, 0.77mg/ml yeast tRNA

SDS-PAGE electrophoresis running buffer: 25mM Tris-HCl, pH8.3, 192mM glycine, 1% SDS (w/v).

SDS-PAGE resolving gel: 10% acrylamide (w/v), 0.27% N,N'-methylene-bis-acrylamide (w/v) (Protogel™, National Diagnostics), 375mM Tris-HCl, pH8.8, 0.1% SDS (w/v), 0.1% ammonium persulphate (w/v), 0.1% temed (v/v).

SDS-PAGE stacking gel: 4% acrylamide (w/v), 011% N,N'-methylene-bis-acrylamide (w/v) (Protogel™, National Diagnostics), 125mM Tris-HCl, pH6.8, 0.1% SDS (w/v), 0.1% ammonium persulphate (w/v), 0.1% temed (v/v).

PBS-T: PBS, 0.05% Tween-20 (v/v).

Blocking buffer (Western): 5% non-fat milk (Marvel) in PBS-T .

2.1.2 Plasmids

Plasmids, subclones, cloning strategies and the creation of promoter contructs and retroviral vectors are presented in detail in the Appendix. However the commercial plasmids with the reporter genes: luciferase and enhanced green fluorescent protein and the retroviral vector backbones employed are mentioned here briefly.

2.1.3 Reporter and Marker Genes

2.1.3.1 Luciferase

The pGL3 Luciferase Reporter vectors from Promega carry the luciferase gene of the firefly (*Photinus pyralis*), which contains a modified coding region for optimised monitoring of the transcriptional activity in transfected eukaryotic cells. Here, the

pGL3-Basic vector (Promega, see plasmid map 8.2.1) without a eukaryotic promoter and enhancer sequences was used for insertion of selected myeloid specific promoter sequences in front of the luciferase reporter gene.

The sensitivity of the luciferase assay provides the basis for fast and quantitative analysis of factors that potentially regulate mammalian gene expression by maintaining relatively low background expression.

2.1.3.2 Green Fluorescent Protein

The gene for the green fluorescent protein (GFP) from the jellyfish *Aequorea victoris*, is a reporter molecule for monitoring gene expression. Its advantage is that it allows the evaluation of transduced cells *in vivo* by direct visualisation under a fluorescence microscope and/or by flow cytometry. In the plasmids pEGFP-N1 (Clontech, see plasmid map 8.4.1) and pIRES2-EGFP (Clontech, see plasmid map 8.8.1) the fluorescent protein is expressed under the control of the immediate early promoter of the human cytomegalovirus (CMV). The eGFP encoding the red shifted humanised mutant was human codon-optimised (Cormack *et al.*, 1996). It contains a chromophore mutation that produces fluorescence 35 times more intense than the wild-type GFP. The 'e' standing for enhanced.

2.1.4 Retroviral vectors

2.1.4.1 pMBΔ

The retroviral vector pMBΔ (see plasmid map 8.3.1) is a derivate of the pBabe series of MLV (Morgenstern and Land, 1990) (5`LTR: 1-1375 and 1965-4125) and carries a modified 3`LTR from pMPSV (1375-1965: 3`LTRΔ) with a deletion of the enhancer. The pMBΔ vector was obtained from Dr. C. Porter, Chester Beatty Laboratories, London. The vector pMBΔ lacks the *pol* and *env* sequences, but carries its the own *cis*-acting encapsidation sequence ($\psi$) located between 5`LTR and the *gag* gene for packaging into viral capsids.

2.1.4.2 Pinco

The retroviral vector PINCO (Grignani et al., 1998) was employed to generate high-titre retrovirus from the Phoenix™ retroviral packaging cells (amphotropic and ecotropic) by transient transfection (Kinsella and Nolan, 1996). The PINCO vector (see plasmid map 8.5.1) contains the full length Moloney LTRs, the extended Ψ sequence and a transcriptional cassette composed of the CMV promoter and the cDNA for the Enhanced Green Fluorescent Protein (eGFP). In addition, the backbone of the PINCO plasmid also contained the EBV oriP and EBNA-1 gene enabling the vector to replicate as an episome. The PINCO plasmid DNA was a kind gift from Prof. Dr. Pier Giuseppe Pelicci (European Institute of Oncology, Milan, Italy)

2.1.5 Mammalian Cell lines and routine culture

All cell lines were maintained at a density between 10^5-10^6cells/ml and cultured at standard condition, 37°C, 5%CO_2 in BIOHIT tissue culture incubators.

2.1.5.1 Suspension Cell Lines

Suspension culture of haematopoietic cell lines, e.g. **HL-60** cells, a human promyelocytic cell line (Collins et al., 1977; Gallagher et al., 1979; Mulder et al., 1981); **U937** cells, a human promyelocytic and histiocytic lymphoma cell line (Sundstrom and Nilsson, 1976); **PLB985** cells, a human myeloblastic leukemia cell line (Tucker et al., 1987); **HEL** cells, a human erythroleukemia cell line (Martin and Papayannopoulou, 1982) and **Jurkat** cells, a human T cell leukaemia cell line (Schneider et al., 1977) were maintained in RPMI-1640 medium (GibcoBRL, Life Technologies) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine with Penicillin-Streptomycin (GibcoBRL, Life Technologies). While **TF-1** cells, a human erythroleukemia cell line (Kitamura et al., 1989) required the addition of 2ng/ml rhGM-CSF to the suspension culture medium.

2.1.5.2 Adherent Cell Lines

Adherent cell lines, e.g. **NIH3T3** cells, a murine fibroblasts cell line (Aaronson and Todaro, 1968); **HeLa** cells, a human cervical carcinoma cell line (Scherer et al., 1953); **293T/17** cells, a derivate cell line of the 293 human embryonic kidney cells (Graham et al., 1977), which expresses the simian virus 40 large T antigen and the 293T cell line named **Phoenix™** a retroviral producer cell line were maintained in DMEM (GibcoBRL, Life Technologies), supplemented with 10% fetal calf serum (GibcoBRL) and Penicillin-Streptomycin (GibcoBRL, Life Technologies).

Cells were passaged at a confluency of 70-80%, e.g. cells were detached from the tissue culture flask on incubation with trypsin-EDTA at standard culture condition for 2min, before the trypsin was inactivated by the addition of culture/growth medium, centrifugation at 1200×g for 5 minutes and then resuspended in the appropriate growth medium.

2.1.5.3 Phoenix™ retroviral producer cell lines

The Phoenix™-ECO and Phoenix™-AMPHO cell lines were obtained from the American Type Culture Collection (ATCC) for non-commercial use through the Nolan laboratory (Stanford University Medical Centre, CA) upon a material transfer agreement:

Name	ATCC Inventory No.	Env
Phoenix™-AMPHO	SD3443	Ampho
Phoenix™-ECO	SD3444	Eco

The Phoenix™ cell lines are based on the 293T cell line (a human embryonic kidney line transformed with adenovirus E1α and carrying a temperature sensitive T antigen co-selected with neomycin). The unique feature of this cell line is that it is highly

transfectable with either calcium phosphate mediated transfection or lipid-based transfection protocols.

The Phoenix™ cell lines are a derivate of the a first generation producer system established by Garry P. Nolan, Warren Pear, and Martin Scott in the laboratory of David Baltimore using 293T cells as a packaging system for helper-free retroviral production (Kinsella and Nolan, 1996; Pear et al., 1993).

The Nolan laboratory made several improvements over the first generations approaches. The Phoenix™-ECO and Phoenix™-AMPHO packaging cell lines were created by placing constructs to be capable of producing *gag-pol* and the envelope protein for either amphotropic or ecotropic viruses into T293 cells. The *gag-pol* genes were introduced with hygromycin as the co-selectable marker, while the genes for the envelope proteins were introduced with diptheria toxin resistance as the co-selectable marker. In addition an IRES-CD8 surface marker was introduced downstream of the reading frame of the *gag-pol* construct to monitor *gag-pol* production by flow cytometry.

The Phoenix™ cell lines offer great advantages over previous stable systems in that they are fully compatible with transient and stable (episomal) retroviral vector gene production and that virus can be produced in just a few days. They also have been extensively tested for helper virus production and established as being helper-virus free by the the Nolan laboratories.

Additionally, a line termed Phoenix™-GP (SD3514) is available (that expresses only *gag-pol*-IRES:CD8-*alpha*) for pseudotyping of retroviral virons with other envelope proteins such as gibbon ape leukaemia envelope, or the vesicular stomatitis envelope protein (VSV-G).

2.1.6 Bacteria

Competent *E. coli* JM109, DH5α, DM1 and SURE® cells as supplied by either Stratagene or Invitrogen were stored in aliquots at -70°C and employed for gene cloning as required.

2.2 Methods

2.2.1 Bacterial growth

E. coli bacterial strains were grown in sterile *Luria-Bertani* medium (LB) or "Terrific Broth" (TB). The selective expansion of *E. coli* carrying plasmid DNA was achieved by growth in the presence of the appropriate antibiotic. *E. coli* carrying plasmids encoding ampicillin resistance (*amp*^{r}) were selected in 100μg/ml ampicillin; *E. coli* carrying plasmids encoding kanamycin resistance gene (*kan*^{r}) were selected in 50μg/ml kanamycin.

2.2.2 Bacterial growth on semi-solid media

E. coli was grown on 15% LB agar plates in the presence of the appropriate antibiotic. Transformed bacteria was spread across the entire plate with a sterile glass rod; bacteria derived from a clonal glycerol stock was streaked in lines with a sterile plastic rod. Plates were incubated upside down for 12-16h at 37°C. Bacteria from individual colonies was picked using a sterile plastic rod and transferred to liquid culture.

2.2.3 Bacterial growth in liquid culture

Individual bacterial colonies were expanded in liquid culture. A sterile culture of LB or TB medium containing the appropriate antibiotic was inoculated with bacteria picked from a single, freshly grown colony and incubated for 12-16h. at 37°C with shaking at 300 cycles/min.

2.2.4 Preparation of frozen glycerol stocks

A one ml aliquot of bacterial culture grown from a single colony was mixed with sterile glycerol to a final concentration of 20% in a 1.8ml cryovial (Nalgene), and stored at -70°C. Short-term storage (3-6 months) at -70°C did not appear to affect the integrity of the sample.

2.2.5 Preparation of competent cells

Treating bacteria cells with specific salt containing solutions, e.g. $CaCl_2$ changes the membrane charge in a way that these cells become temporarily permissive to the transformation of DNA molecules.

Competent *E. coli* JM109, DH5α, DM1 and SURE® cells were prepared aseptically. Bacteria was streaked onto LB agar (without antibiotic) and incubated at 37°C for 14-16h. A single colony was picked and incubated in 100ml LB medium in a 1 litre flask at 37°C with shaking at 300 cycles/min. until the optical density (OD) of the culture at 550nm reached 0.65–0.75OD units. The culture was harvested by centrifugation at 5000×g at 4°C. The bacterial pellet was resuspended in ice-cold 20ml of 100mM $CaCl_2$ solution and incubated on ice for 20min. Cells were recovered by centrifugation at 5000×g, 4°C for 10 min., resuspended in 4ml ice-cold 100mM $CaCl_2$ solution 50ml of original culture. For the preparation of permanent competent cells 1ml of sterile Glycerol was added to the cell suspension, vortexed of 0.5min and transferred immediately to aliquots of 200µl per eppendorf tubes, which were shock frozen in liquid Nitrogen and stored at -70°C.

2.2.6 Preparation of plasmid DNA

Plasmid DNA was amplified by growth of transformed bacteria in liquid culture and purified on a small 'mini-prep' scale from a 5ml culture or on a large 'maxi-prep' scale from a 250ml culture. For optimal reproducible DNA quality and purity, the solutions and protocol of the commercial available Plasmid DNA Purification Kits from QIAGEN Ltd, UK or HYBAID UK were essentially followed.

2.2.7 'MiniPrep' isolation and purification of plasmid DNA

Small scale purification of plasmid DNA from bacteria culture was performed using the HYBAID RECOVERY™ Quick Mini Spin Kit (cat.# RY-10250, HYBAID, UK),

following manufacturer's instructions. The protocol involves isolation of plasmid DNA by alkaline/SDS lysis method and a subsequent spin of the neutralised lysate in a spin column, where the plasmid DNA is bound to an adsorption matrix.

Briefly, cells were harvested from 1-3ml of bacterial culture by centrifugation at maximum speed (approx. 10000×g) in a tabletop microfuge, like all following centrifugation steps. All traces of medium were removed carefully, and the bacteria pellet was resuspended in 210µl Cell Suspension Solution (HYBAID) by vortexing until the suspension was homogeneous. Then, 210µl Cell Lysis Solution (HYBAID) was added and mixed thoroughly by inverting the tube several times and left for 5min at RT for cell lysis. After this time, 280µl Neutralisation Solution (HYBAID) were added and mixed immediately by inverting the tube until a homogenous phase was obtained. To clear the solution for column loading the mixture was centrifuged at 10000×g for 10min before the supernatant was transferred into a spin column (HYBAID) that was placed into a 2ml receiver tube (HYBAID). The tube was centrifuged for 1min and the flow-through was discarded. Incubation with 500µl of solution GX (HYBAID) for 1 min, centrifugation and disposal of the flow-through was recommended as an optional column wash for nuclease-rich bacteria. Otherwise the column was subjected to column wash by addition of 700µl of reconstituted Wash solution (HYBAID) and centrifugation at maximum speed for 1min. After the flow-through was discarded, it was essential to remove all residual solution in an additional micro centrifugation step. Finally, the plasmid DNA was eluted by placing the column in a new 1.5ml microfuge tube and adding 75µl of sterile water (or 10mM Tris-HCl, pH8.0 buffer) directly onto the center of the silica matrix of the spin column before centrifugation at 10000×g for 2min was performed. DNA dissolved in water was stored at minus 20°C.

2.2.8 'MaxiPrep' isolation and purification of plasmid DNA

Large scale purification of plasmid DNA from bacteria culture was performed using the HYBAID RECOVERY™ Quick Flow Maxi Kit (cat.# RY-14020, HYBAID, UK), following manufacturer's instructions. The protocol involves isolation of plasmid DNA by alkaline/SDS lysis method and is based on gravity flow in combination of an anion

exchange resign, which is supplied in Quick Flow columns (HYBAID) and results in ultra pure (CsCl grade) plasmid DNA.

Briefly, a 100-250ml bacterial culture were incubated overnight for 12-14h and harvested at an density of approximately 1×10^9 cells/ml LB medium by centrifugation (at 6000×g in a Becton Dickinson Centrifuge). All traces of medium were removed carefully, and the bacteria pellet was resuspended in 10ml Resuspension Solution (HYBAID) by vortexing until the suspension was homogeneous. Then, 10ml of Cell Lysis Solution (HYBAID) was added and mixed gently by inverting the tube several times and left for cell lysis for not more than 5min at RT to avoid irreversibly denaturing the DNA. Meanwhile the Quick Flow columns were equilibrated with 30ml of Equilibration Solution (HYBAID) by gravity flow-through. 10ml of Neutralisation Solution (HYBAID) were added to the cell lysis mixture and mixed immediately by inverting the tube several times. To clear the solution before column loading the mixture was centrifuged at 15000×g for 10 min. The supernatant was transferred promptly to the equilibrated column to allow the lysate to run trough by gravity flow. The column washed by addition of 60ml of Column Wash Solution (HYBAID) and allowed to empty by gravity flow. Finally, adding 15ml of Elution Solution (HYBAID) to the column eluted the plasmid DNA. The precipitation of the DNA was carried out by adding 0.7 volumes isopropanol (ultra grade) (10.5ml). The DNA was harvested by centrifugation at 15000×g for 30min. The DNA pellet was washed twice with 70% ethanol, air dried for 10min and re-dissolved in a suitable volume of buffer. DNA dissolved in water was stored at -20°C.

2.2.9 Quantification of nucleic acid by spectrophotometry

The purity and concentration of nucleic acid solutions were quantified by optical density (OD) measurement at wavelengths of 260nm and 280nm with a Philips PU8620 spectrometer or GENQUANT from Pharmacia. Absorption of 1 OD_{260nm} unit equals a concentration of 50μg/ml for double stranded DNA, 40μg/ml for single stranded DNA or RNA and 33μg/ml for oligonucleotides. The OD_{260nm} / OD_{280nm} ratios of 1.8

and 2.0 indicate the purity of samples for solutions of DNA and RNA respectively, which were calculated automatically using the GENQUANT spectrometer.

2.2.10 Quantification of nucleic acid by ethidium bromide fluorescence

The concentration and purity of diluted DNA solutions (<250ng/ml) was carried out by agarose gel electrophoresis and estimated by comparison of the intensity of fluorescence of DNA under shortwave ultraviolet (UV) radiation (300nm) with that of known amounts of 1kb or 1kb plus DNA ladders (GibcoBRL Life Technologies).

2.2.11 Restriction enzyme digestion of DNA

All restriction enzymes and their buffers were obtained from Promega. DNA was digested according to manufacturer's instructions using 1× supplied restriction enzyme buffer and an excess of enzyme (5-10U/μg DNA). Unless otherwise stated all digestions to produce fragments for subcloning were performed in a total volume of 50μl reaction mix with 5-10μg plasmid DNA and incubated at 37°C for 1-2h in the appropriate 1× digestion buffer and presence of restriction enzyme (5-10U/μg DNA).

The digestion of genomic DNA (10-25μg) was carried out under the same condition, but with an extended incubation period of 14-16h and the addition of 100μg/ml bovine serum albumin (BSA) and 4mM spermidine whilst 10U/μg DNA restriction enzyme was used. The successful completion of digestion was confirmed by agarose gel electrophoresis of approximately 10-20μl of the digested sample.

2.2.12 Analysis of nucleic acid by Agarose Gel Electrophoresis

1-2% Agarose (GibcoBRL) (w/v) gels were cast in 1× TAE supplement with 500μg/ml ethidium bromide. DNA samples were prepared in 1× DNA loading dye (6× stock solution), and loaded onto the gel along with DNA markers, 1μg of 1kb or 1kb plus DNA ladders (GibcoBRL Life Technologies). Electrophoresis was performed in 1× TAE electrophoresis buffer and a voltage gradient of 15-20V/cm. Large DNA

molecules (>10kb) were separated on 0.8-1.0% agarose; molecules ranging from 1.3-10kb were separated on 1% agarose, whilst molecules fragments of less than 1.3kb in size were separated on 2% agarose. After electrophoresis the analysis of the gel was carried out and examined under UV transillumination.

2.2.13 Purification of DNA

For rapid clean-up of oligonucleotides and DNA restriction fragments between 100bp and 15kb from enzymatic reactions (e.g. labelling, dephosphorylation, restriction and tailing) the commercial available QIAquick Spin Kits for 'Nucleotide Removal' and 'Gel Extraction' from QIAGEN were used following manufacturer's instructions. The technique involves pH-dependent DNA adsorption to a silica-membrane with a high concentration of chaotropic salts (Vogelstein and Gillespie, 1979) while contaminants were washed out and pass through a spin-column during the microcentrifugation process. Finally, the pure DNA was eluted in Tris buffer or water.

2.2.14 Excision of DNA fragments from agarose gel

DNA restriction fragments were separated by agarose gel electrophoresis and visualised by illumination under shortwave UV radiation (300nm). Fragments were individually excised from the gel with a clean, sharp scalpel blade and transferred into an eppendorf tube. DNA purification was carried out following essentially the instruction of the 'QIAquick Gel Extraction Kit Protocol' (QIAGEN). Briefly, the approximate weight of the gel slice was determined and 3 volumes of Buffer QG (QIAGEN) to 1 volume of gel (100mg ~ 100µl) were added to the sample. The gel slice was dissolved by incubation at 50°C for about 10min, and was vortexed until the gel was completely dissolved. The provided Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding, which can be corrected by adding 10µl of 3M sodium acetate. To allow optimal DNA binding 1 gel volume of isopropanol was added to the sample, mixed and transferred to a QIAquick spin column in a provided 2ml collection tube. The flow-

through was discarded after 1 min of centrifugation and by adding new 500µl Buffer QG in a second spin all traces of agarose were removed. The column was washed with 750µl of Buffer PE (QIAGEN), the spin flow-through was discarded and any residual ethanol from the Buffer PE was removed completely by an additional 1 min spin at 10000×g. Finally, the spin-column was placed into a clean 1.5ml microfuge tube and the DNA was eluted in 30-50µl water or 10mM Tris-HCl (pH8.5) buffer. For subcloning DNA fragments were eluted in absence of a buffering agent and stored at -20°C.

2.2.15 Purification of DNA fragments from aqueous solution

To remove primers <10 bases, enzymes, salts, and unincorporated nucleotides the 'QIAquick Nucleodide removal Kit Protocol' (QIAGEN) was essentially followed. Briefly, 10 volumes of Buffer PN (QIAGEN) were added to the reaction sample and mixed. For example, 500µl of Buffer PN was added to a 50µl reaction sample and transferred into a QIAquick spin column in a provided 2-ml collection tube. To bind DNA, the first spin was carried out at 6000×g for 1 min. The flow-through was discarded and the column was washed by adding 750µl of Buffer PE (QIAGEN) followed by another spin at 6000×g. Residual ethanol from Buffer PE was completely removed with an additional centrifuge step at 10000×g. Finally, the spin-column was placed into a clean 1.5ml microfuge tube and the DNA was eluted in 50-100µl water or 10mM Tris-HCl (pH8.5) buffer. For subcloning DNA fragments were eluted in absence of a buffering agent and stored at -20°C.

2.2.16 Preparation of vector DNA for subcloning

Vector DNA (5-10µg) was linearised by restriction enzyme digestion and if necessary separated from unwanted DNA fragments by agarose gel electrophoresis. The DNA purification was carried out as described above with the QIAquick Spin Kits from QIAGEN. Linearised vector DNA with cohesive termini was phosphatase-treated in order to prevent self-ligation.

2.2.17 Dephosphorylation of DNA fragments

To prevent self-ligation of purified vector DNA the 'Shrimp Alkaline Phosphatase Dephosphorylation Protocol' from United States Biochemical (USB) was essentially followed. Briefly, the shrimp alkaline phosphatase has approximately the same specific activity as the calf intestine enzyme (800-1000U/mg at 25°C, pH9.6) but, unlike the calf enzyme, it is completely and irreversibly inactivated in Tris buffers at pH8.0-8.5 by simply heating for 15min at 65°C. The rate of removal of the terminal 5`phosphate from double-stranded DNA depends on the structure of the terminus. Termini with 5` protruding ends are more reactive than those with blunt-ends or those with 5`-recessed ends. The reaction rate also depends on the temperature and magnesium concentration. Vector DNA (1-5µg) was incubated at 37°C for one hour in 1× Shrimp Alkaline Phosphatase Buffer (USB, supplied 10× Buffer: 200mM Tris-HCl, pH8.0 and 100mM $MgCl_2$) and the recommended enzyme concentration in a total volume of 50µl. For a typical reaction using 1.0pmol of DNA termini (2.5µg of a 3kb plasmid), the following minimum amounts were found to be effective: 5`-protruding (0.1U), blunt (0.2U) and 5`-recessed (0.5U). Shrimp Alkaline Phosphatase treated DNA was purified after 15min enzyme heat-inactivation at 65°C with QIAquick Nucleotide Removal Kit (QUIAGEN). The extent of dephosphorylation was assessed by ligation and transformation of dephosphorylated vector. Successfully dephosphorylated vector should not religate or consequently produce no colonies. The concentration of vector DNA was estimated by ethidium bromide fluorescence from 5µl DNA aliquot.

2.2.18 Preparation of insert DNA

Insert DNA was prepared by restriction enzyme digestion and agarose gel electrophoresis. DNA was purified from agarose following the 'QIAquick Gel Extraction Kit Protocol' (QIAGEN) and was resuspended in 50µl of water. The concentration of insert DNA was estimated by ethidium bromide fluorescence from 5µl DNA aliquot.

2.2.19 Ligation

To join two strands of purified vector DNA and insert DNA between the 5`-phosphate and the 3`hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration the T4 DNA Ligase (Promega, Cat.#M1794, 10-20U/µl) was employed. In order to avoid exposure to frequent temperature changes and consequently degradation of the ATP and DTT in the Ligase 10× Buffer the T4 DNA Ligase and its Ligase 10× Buffer (Promega) was stored in aliquots at -20°C and on ice at all times during preparation of the ligation reaction sample.

The cloning of an DNA insert into a plasmid vector was performed using a molar ratio of vector : insert DNA of 1 : 3 and 1 : 5 by applying the following formula:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of} \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

The typical ligation reaction was about 100-200ng of vector DNA with the appropriate amount of insert, mixed with 2µl of ligase 10× Buffer and 1µl T4 DNA ligase (Promega, 10-20U/µl), which was adjusted to a final reaction volume of 20µl with nuclease-free water. To allow annealing and ligation of the DNA fragments, the ligation itself was carried out at the optimal incubation temperature for T4 DNA ligase, which is 16°C and recommended for efficient ligations. However, ligase is active at a broad range of temperatures, and for routine purposes such as subcloning, convenience often dictated incubation time and temperature, that ligations performed at 4°C overnight or at room temperature for 30min or a couple of hours usually worked well too.

Only 10µl of the ligation reaction volume was used for the transformation of competent *E.coli* cells.

2.2.20 Transformation of competent cells by heat-shock

Subcloning efficient JM109, DM1, DH5α or SURE® competent *E.coli* cells were used for transformation of plasmid DNA. Frozen competent *E.coli* cells were thawed on ice. 10µl of a ligation reaction mixture or about 50-100ng plasmid DNA was mixed with 100µl of competent cells and left on ice for 10min. After this time, cells were subjected

to a heat-shock in a preheated water bath at 42°C for about 1-1½ min and immediately returned on ice for 3 min. 1ml of LB medium (without any antibiotics) was added, and the transformation mixture was incubated at 37°C for 1h. Transformed bacteria cells were harvested by centrifugation at 6000×g for 5min, the supernatant was removed and the bacterial pellet was resuspended in remaining media. The total volume was then plated onto 15% LB agar plates containing the appropriate antibiotic. The plates were incubated upside-down at 37°C for 14-16h. Positive and negative control transformation reactions containing closed circular plasmid DNA and linear DNA were performed in parallel. Transformation efficiency was assessed by performing a test transformation with closed circular plasmid DNA. The number of colonies produced using 1, 10 and 100ng DNA provides an estimation of colony forming units/μg.

2.2.21 Transformation of electro-competent cells by electroporation

Successful ligation and transformation efficiency is subject to many variables, e.g. concentration, ligation ratio, termini configuration (e.g. cohesive or blunt-end ligation), the permeability of the membrane of the competent bacteria and the size of the plasmid DNA. To eliminate the the latter, it was sometimes necessary to perform transformation of ligation DNA by electroporation.

This was carried out by as follows: 50μl electro-competent DH5α cells were mixed with 10μl ligation reaction sample and transferred further in a chilled electroporation cuvette (BIORAD) stored on ice. The electroporation was performed at 25μF, 2.5kV and 200Ω and immediately placed on ice again, before 1ml of LB medium (RT) was added and incubated at 37°C for 1h. Transformed cells were harvested by spinning and plated onto an 15% LB agar plates containing the appropriate antibiotic. Controls were performed as described above for heat-shock transformation.

2.2.22 Analysis of recombinant bacterial transformants

Single colonies were picked and transferred aseptically into 2-5ml LB medium containing appropriate antibiotic. The liquid culture was incubated at 37°C for 12-16h

with shaking at 300×g. For analysis an aliquot of 1-2 ml was taken and the extraction and purification of plasmid DNA was performed following essentially the HYBAID RECOVERY™ Quick Mini Spin Kit Protocol (HYBAID UK). Plasmid DNA was analysed by restriction enzyme digestion and agarose gel electrophoresis. Positive identified clones were aliquoted for glycerol stock and/or stored at 4°C for not more then 1-2 days before a expansion of the bacteria culture was performed for 'MaxiPrep' purification of plasmid DNA.

2.2.23 Transfection of mammalian Cells

Various transfection methods were employed to deliver plasmid DNA into mammalian cells. We used transfection by electroporation for suspension cell lines and transfection by LID complex or transfection by calcium phosphate precipitation for adherent cell lines.

2.2.23.1 Transfection of U937 and HL-60 cells by Electroporation

The conditions used to electroporate U937 and HL60 cells for transient expression are essentially described in (Pahl *et al.*, 1991), but were applied in a slightly modified way, which turned out to be more efficient in our hands.

Briefly, suspension cells were diluted to 10^5cells/ml and incubated in fresh RPMI-1640 medium 16-24h prior to transfection. At the time of transfection, the cells were harvested by centrifugation at 500×g for 5min at room temperature (RT). Cells were washed twice in 4°C cold RPMI-1640, containing neither fetal calf serum (FCS) nor L-glutamine. Cells were resuspended at 2.5-3×10^7cells/ml in RPMI-1640, and a 450µl aliquot was added to a chilled sterile electroporation cuvette (BioRad). Then, in total 20µg of plasmid DNA (i.e. to determine the relative transfections efficiency, the experimental luciferase-expressing plasmids were routinely co-transfected with pEGFP-N1 plasmid DNA at the same amounts) were dissolved in 50µl RPMI-1640 (or sterile water) was added to the cell suspension in the cuvette and left on ice for additional 15min before the electroporation was performed with 200V at 960µF. The cuvette was returned to the ice bucket for further 5min before the cells were transferred

and resuspended in 20ml of cold complete RPMI-1640 media and then cultivated at 37 °C for cell recovery in the cell culture incubator.

For cell induction experiments with PMA, the cell suspension above was split 50:50 by transfer of 10ml to a 50ml tissue culture flask with added PMA (1ng/ml) before both flask (±PMA) were returned to the incubator to recover for 24-48 hours until harvested for luciferase assays at various times.

The transfection efficiency was determined by fluorescence microscopy using red and green filters on an epifluorescent microscope (Nikon) by counting a minimum of 10^2 cells per transfection with a haemocytometer. The percentage of eGFP positive cells were used to normalize the luciferase RLU/mg protein to the number of cells transfected.

2.2.23.2 Transfection of tissue culture cells with LID complex

The following transfection method with Lipofectin intergin targeting peptide : DNA complexes (LID) (being developed and optimised in our laboratory at the time) was made available to us by Drs. Steven Hart and Aima Uduehi (see (Hart et al., 1994; Hart et al., 1995; Hart et al., 1997; Hart et al., 1998; Uduehi et al., 2003).

To begin with, the integrin targeting peptide (the AS sequences for peptide 6 and 8 are listed in the table futher down) required stock solution preparation: This cyclisation of peptide was carried out by dissolving 2mg of peptide to a concentration of 0.1mg/ml in OptiMEM® (Gibco Life Technologies) in a 20ml conical flask, which was left at 4°C overnight before it was sterile filtered, aliquoted and stored at –70°C until use.

No.	AS sequence	Supplier
Peptide 6	$[K]_{16}$GACRRETAWACG	Zinsser Analytic, Maidenhead, Kent, UK
Peptide 8	$[K]_{16}$GACQIDSPCA	Zinsser Analytic, Maidenhead, Kent, UK

The LID transfection complex was formulated at a ratio of 0.75 : 4 : 1 of Lipofectin : Intergin peptide : DNA. This ratio is applicable when peptides 6 or 8 are

used. For other peptides the amounts may need to be altered so that complexes with a net positive charge of 6.8 are formed. Unless otherwise stated peptide 6 was used in our experiments, which proved to be best for transfection of haematopoietic cells (Drs. Steven Hart and Aima Uduehi, personal communication (Hart et al., 1997; Hart et al., 1998; Uduehi et al., 2003)).

First stock solutions of the LID complex components were prepared: lipofectin (supplied as a 1mg/ml solution, Gibco Life Technologies) was diluted to 0.75µl/100µl OptiMEM® (Gibco Life Technologies), plasmid DNA was diluted to 1µg/100µl OptiMEM® and cyclised peptide at 0.1mg/ml in OptiMEM® was used. The transfection complexes were made sufficient for 1µg plasmid DNA per well in a 48 well plate, while the plasmid DNA of the experimental luciferase-expressing plasmids were mixed with pEGFP-N1 plasmid DNA at the same amounts to assess the relative transfections efficiency. The relative amounts for complex formation and order of adding to the formulation tube were: (1) lipofectin: 0.75µl (0.75µg) in 100µl OptiMEM®, (2) peptide (0.1mg/ml in OptiMEM®): 40µl (4µg) and plasmid DNA (1µg/100µl): 100µl (1µg) were mixed and left under ambient conditions between 30min and 2h to allow the formation of the LID complexes.

Tissue culture cells for transfection were prepared as follows: 16-24h prior to transfection, haematopoietic cells, e.g. U937 or HL60 were diluted to 1×10^5cells/ml and incubated in fresh RPMI-1640 medium. At the time of transfection, the cells were harvested by centrifugation at 1200×g for 5min at room temperature (RT) and washed twice in OptiMEM®. The cell concentration was determined by using a haemocytometer, resuspended and seeded at a density of 5×10^4 cells/300µl OptiMEM® per well into 48 well plate. 200µl LID complex, formulated as described above, was added to each well making up a final concentration of 1µg plasmid DNA per 500µl/well. Transfection of cells occurred during 4h of incubation in the BIOHIT incubator under standard tissue culture conditions (37°C, 5%CO_2). After this time transfection excess medium was removed and replaced with 500µl fresh complete RPMI-1640 growth medium.

The transfection efficiency was determined by fluorescence microscopy using red and green filters on an epifluorescent microscope (Nikon) by counting a minimum of 10^2 cells per transfection with a haemocytometer. The percentage of eGFP positive cells were used to normalize the luciferase RLU/mg protein to the number of cells transfected. The analysis of the transient reporter gene expression assay was carried out at various times.

2.2.24 Luciferase assay

Luciferase activity in cell extracts was measured using the luciferase assay system (Promega UK Ltd., Southampton, UK) as described by the manufacturer.

First, cells were rinsed in 1× PBS and then re-suspended in 100µl of 1× Reporter Lysis Buffer (Promega) and lysed by incubating for 15min at 4°C. Samples were then subjected to one freeze-thaw cycle and the cell suspension pelleted by centrifugation at 1300×g for 5min. The supernatant was then assayed for luciferase activity using the Luciferase Assay Kit (Promega), e.g. 20µl of cell lysate was mixed with 100µl of Luciferase Assay Reagent and measured in the Anthos Lucy microplate luminometer/photometer (Labtec Intl., Sussex, UK).

In the luciferase assay, luciferin and Mg^{2+}ATP are added to cellular extracts which induces the production of light. Total light emission of each sample was measured for 10 seconds as well as the protein content in corresponding samples was analysed using the Bio-RAD Protein Assay Kit (Bio-Rad Laboratories, Herts., UK) with BSA as a standard.

All data are expresses as RLU (relative light units) standardised to protein content (RLU/mg) and corrected for transfection efficiency, e.g. the percentage of eGFP positive cells were used to normalize the luciferase RLU/mg protein to the number of cells transfected.

2.2.25 Passage and Cultivation of Phoenix™ cells

The Phoenix™ packaging lines were maintained in GM. Care was taken to ensure that cells did not reach confluency as this will reduce transfection efficiency in the short term. To keep these cells in optimal healthy condition, a 1:5 "split" of a 70-80% confluent 100mm plate into new plates was performed every 2-3 days. This was carried out by aspirating old growth medium, then rinsing cells gently with 1× PBS (without Ca^{++} or Mg^{++}) and application of trypsin (0.05% trypsin/0.53mM EDTA) until the cells detached. Trypsinisation was inactivated with GM prior to re-seeding cells into fresh medium.

2.2.26 Phoenix™ drug selection

Every few month the Phoenix™ cells were selected for one week in Hyrgromycin B (Sigma-Genosys, Poole, UK) at 300µg/ml and Diphtheria Toxin from *corynebacterium diphtheria* (Sigma) at 1-2µg/ml final concentration on cells. After one week of selection Phoenix™ cells produce high titre virus, which can be repeated every three month after a titre drop occurs. Cells can be analysed and sorted by FACS for expression of CD8 (a proxy measure of *gal-pol* in this cell line) and for surface expression of envelope protein with 83A25 antibody (Hybridoma) as descript in (Evans et al., 1990).

2.2.27 Cell Freezing

Exponentially growing cells can be stored frozen for many years using cryoprotective agent such as DMSO and glycerol. Subconfluent cells were washed with PBS, then trypsinised for a few minutes and inactivated by adding fetal calf serum (FCS). The cells are washed by centrifugation at 500×g for 5 min and resuspended in appropriate volume of freezing media (10% DMSO, 90% FCS) at 2-4 × 10^6cells/ml. Alquotes of resuspended cells were then transferred immediately into a sterile 2ml cryogenic vial. These freezing vials were then placed in a special freezing container (Nalgene® Mr. Frosty) containing isopropyl alcohol, to provide the critical and repeatable 1°C/min

cooling rate required for successful cryopreservation of cells at -80°C overnight before being transferred to liquid nitrogen the following day.

It was recommended to freeze multiple vials of each producer cell line after first receiving and expanding them. This ensured a ready supply of backup vials to allow uniform virus production over several years. If the cells are subjected to selection media, this should not be applied until after the first passage.

2.2.28 Thawing 293 cells and Derivatives

After removal of the freezing vial from liquid nitrogen cells were thawed rapidly at 37°C. 1ml of GM was added immediately to the freezing vial. This solution was gently transferred into a 15ml sterile conical screw cap tube and suspended in further 5ml of GM by gently mixing the tube to allow osmotic equilibration. After adding 10ml of GM, the tube was closed, inverted several times and centrifuged at 500×g for 5min. Supernatant was removed, and cell pellet resuspended in GM before transferred to a 100mm (or 150mm) tissue culture dish and grown in a 37°C degree incubator containing 5% CO_2

2.2.29 Transfection of tissue culture by Calcium Phosphate Precipitation

The calcium phosphate transfection method for introduction of DNA into mammalian cells is based on the formation of a calcium phosphate-DNA precipitate. The calcium phosphate ($CaPO_4$) is thought to facilitate the binding of the DNA to the cell surface. It is believed that the DNA then enters the cell by endocytosis. The method was first developed by (Graham and van der Eb, 1973) and was later modified by (Wigler et al., 1977). The procedure is routinely used to transfect a wide variety of cell types for either transient expression or for the production of stable transformants. The DNA is mixed directly with a concentrated solution of $CaCl_2$. This is then added dropwise to a phosphate buffer to form a fine precipitate. Aeration of the phosphate buffer while adding the DNA-$CaCl_2$ solution helps to ensure that the precipitate which forms is as

fine as possible. This is important because clumped DNA will not adhere to or enter the cell as efficiently.

Essentially, calcium phosphate transfection solutions were used as supplied by Invitrogen (Paisley, UK) and employed as advised by the manufacturer. For example, for transfection of retroviral packaging lines (2.1.5.3) by $CaPO_4$ precipitation the following procedure was carried out:

Unless otherwise noted, all conditions are described for 150mm plates. 18-24h prior to transfection, Phoenix™ cells were seeded at 5×10^6 cells per 150mm plate were seeded in 20ml of GM, which should be approximately 70-80% confluent at the moment of transfection. Just prior to transfection the medium was replaced with 20ml of fresh GM containing 25μM chloroquine, which has been shown to enhance transfection efficiency by inhibiting DNA degradation by lysosomes (Luthman and Magnusson, 1983).

The transfection cocktail was prepared in a round bottomed tube (BD Falcon™) by adding 20 to 40μg DNA to sterile H_2O such that the final volume is 900μl. Then 100μl 2.5M $CaCl_2$ was added and mixed, before drop-wise addition of 1ml 2× HBS solution (HEPES buffered saline, pH 7.05) by bubbling and mixed thoroughly for about 0.5min with a pipette-aid to assist the formation of a very fine precipitate. This solution was then added immediately (within 1-2min) in single drops to the cells and agitated gently to ensure uniform mixing. Cells were returned for 6-12h to the incubator at 37°C, before media was replaced with 20ml of fresh GM for cell culture expansion (or puromycin selection) before harvesting the retroviral supernatant.

2.2.30 Production of high titre retroviruses by transient transfection

Producer cells transfected with retroviral constructs based on the PINCO vector backbone (carrying a puromycin selectable marker) were selected by culture in medium supplemented with 1μg/ml puromycin for at least 3-4 days and re-seeded as required.

In general transient viral supernatant was collected 24-48h post-transfection following incubation of transfected packaging cells covered with a minimal amount (e.g. ~10ml/150mm dish) of fresh GM media at 32 °C for 8-24h before harvesting.

Supernatant was filtered through a 0.45μm filter and used immediately for transduction or stored at -70 °C until required.

2.2.31 Estimation of retroviral titre by FACS analysis

Retroviral titres from supernatant of transient and episomal stable transfections with PINCO vector and its modified derivates carrying the EGFP marker gene were harvested from the retroviral packaging cell lines (Phoenix™ amphotropic and ecotropic) were determined on NIH 3T3 cells.

NIH 3T3 cells were seeded at a density of exact 5×10^4 cells per well in a 24 well plate. Then 1ml of an infection cocktail, containing retroviral supernatants ranging from 1μl to 100μl, and GM supplemented with 8μg/ml polybrene was added to individual wells. Infection was carried out overnight at 37°C. The following day, the infection cocktail was removed and 1ml of fresh medium was added. Cultivation of transfected cells were carried out at standard culture conditions for a further 48h. Then the cells of each individual well were trypsinised, washed twice in PBS, and fixed with Leucoperm™. To determine the expression of eGFP in the NIH 3T3 cells the cells were then assayed and analysed by flow cytometry (FACS). Scatter properties of uninfected NIH 3T3 cells were determined and retrovirally transduced cells were compared for number of fluorescing cells. Retroviral titres were calculated by plotting the percentage of fluorescing cells against the volume of viral supernatant used. Retroviral titres were then determined by calculation of the gradient obtained.

2.2.32 Estimation of retroviral titre by Northern dot blotting

To estimate the amount of viral RNA species from viral vector constructs carrying the luciferase gene in cell-culture supernatants, the QIAamp Viral RNA Mini Kit (QIAgen) was employed for the purification of viral nucleic acids from supernatants by following

manufacturer's instructions with QIAgen provided buffers and solutions (2.2.32.1). After transfer of viral RNA onto nitrocellulose membrane and hybridisation with luciferase radio labelled probe (2.2.32.2), and the quantitative analysis of the autoradiographic dot-blots image was carried out by densitometry using a BioImage system (Millipore).

2.2.32.1 Northern dot blotting: RNA Purification

The QIAamp Viral RNA Mini Kit combines the selective binding properties of a silicagel-based membrane with the speed of microcentrifugation for simultaneous processing of multiple samples at the same time. Briefly, at first 140µl per sample of virus containing cell-culture medium supernatant - harvested from retroviral producer cells as described above - was subjected to lysis and denaturation conditions by mixture with 560µl of AVL buffer (containing chaotropic salts and detergent with added carrier RNA at 5.6µg per 140µl sample). In this initial step, RNAses are inactivated and intact viral RNA isolated, while the addition of carrier RNA not only enhances binding of viral RNA to the silica membrane but also reduces the chance of viral RNA degradation. For this viral particle lysis to complete, the solution was mixed by pulse-vortexing for 15s and left at room temperature to incubate for 10min. Then the buffering condition was adjusted for optimal binding of the viral RNA to the QIAamp membrane by adding 560µl of ethanol (96-100%) to the sample, which was mixed again by pulse-vortexing for 15s. After a brief centrifuge spin, to remove any drops from the inside of the lid, 630µl of the sample was loaded onto the QIAamp Mini spin column and subjected to centrifugation at 6000×g for 1 min. This step was repeated with the remaining 630µl of sample. The adsorbtion of the RNA to the QIAmp silica membrane occured during pass through of the sample solution. The pass through was discarded each time. Then any contaminates were washed out using 500µl of different washing buffers (AW1 and AW2) in two steps. To eliminate any chances of possible residual buffer carry over, QIAmp Mini column was subjected to additional 10000×g for 3min. Finaly, the QIAamp Mini column was placed into a 1.5ml microcentrifuge tube (Eppendorf) to collect the silica membrane bound RNA, now free of any protein,

nucleases, contaminants and other inhibitors. Initialy, 60µl of RNase free buffer (AVE) was added onto the silica membrane and left for 1 min incubation time, before centrifugation at 6000×g for 1 min to collect the retroviral RNA. For dot blots this was repeated with the addition of another 80µl of RNase free buffer (AVE) to a total volume of 140µl ready for direct use or storage at -70 °C until required.

2.2.32.2 Northern dot blotting: Hybridisation with radio-labelled probe

For dot blots, a 100µl aliquot per sample of the retroviral RNA purified as described above was spotted under vacuum onto the surface of a nylon membrane Hybond™-N (Amersham, Buckingham, UK) using a dot blot manifold (GIBCO-BRL, Gaithersburg, MD, USA). The nylon membrane was cross-linked with ultraviolet light using a Stratalinker (Stratagene Corp., La Jolla, CA, USA) and hybridised with a α-^{32}P-labeled luciferase cDNA probe overnight.

The radio-labelled probe was generated with the help of the Prime-It® II Random Primer Labelling Kit (Stratagene) following the manufacturer's protocol, e.g. 20-100ng of luciferase cDNA fragment in a total volume of 24µl were mixed with 10µl of oligonucleotide primer and denaturated at 95°C for 5min. After addition of 10µl of 5× dATP buffer and 5µl of [α-^{32}P]-dATP (3000 Ci/mmole) and 1µl of Exo-Klenow polymerase (5U/µl) the labelling reaction was performed for 30min at 37°C. Un-incorporated nucleotides were removed by purification of the labelled probe using MicroSpin™ S-200 HR columns (Amersham, Buckingham, UK). After resuspension of Sephacryl resign by pulse vortexing and removal of the bottom closure, the MicroSpin™ column was centrifuged down at 735×g for 1min and then placed into a new 1.5ml Eppendorf microcentrifuge tube. Then the labelled probe was added onto the resin and eluted at 735×g for 2min.

After pre-hybridisation of the dot plot membrane placed in a hybridisation glass bottle (Hybaid, Thermo Scientific) with hybridisation solution for 1h at 68°C under rotation in a hybridisation oven (Hybaid, Thermo Scientific), the radio labelled luciferase probe was denaturated at 95°C for 5min and added to the pre-hybridisation mixture.

After hybridisation at 68°C overnight, the labelled membrane was washed once with 2× SSC 0.1% sodium dodecyl sulphate (SDS) at room temperature for 10min and twice in 0.1× SSC, 0.1% SDS at 50°C for 20min each. Once the dot blot was air dried, it was transferred to an X-ray film cassette, covered with cling film (SARAN™ wrap) and exposed to autoradiography using X-ray film (Agfa, EC) with intensifying screens at -70 °C for upto 3 days.

2.2.33 Retroviral transduction of cell lines

Various cell lines were transduced according to standard protocols. Virus containing media was added to cell lines in the presence of 4μg/ml polybrene. To enhance co-localisation of virus and cells, several rounds of infection (spinoculation) were carried out. This was done by repeated addition of virus at 6-8h intervals followed by centrifugation at 2500×g for 90min. After each spinoculation, the transduced cells were returned to the incubator for continous culture at 37°C.

2.2.34 Bone marrow isolation, transduction and transplantation

On day 1, 8-12 weeks old donor mice were injected intraperitoneally (i.p.) with 5-fluorouracil (5-FU, 3mg/kg in normal serum, Roche) to enrich for and activate the BM stem cell proliferation. On day 3 (after 48h) donor bone marrow stem cells (BM) were harvested from femur and tibia by flushing with culture medium using a 3ml syringe (BD Biosciences). The isolated cells were then cultured at a density of 1×10^6 cells/ml in a 6-well-plate in RPMI (Invitrogen Life Technologies) containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen Life Technologies), 100U/ml streptomycin/penicillin (Invitrogen Life Technologies), and 2 mmol/l L-glutamine (Invitrogen Life Technologies) for 48h in the presence of 100ng/ml recombinant human interleukin-6 (IL6, PeproTech), 100ng/ml recombinant murine stem cell factor (mSCF, PeproTech) and 50ng/ml murine *fms*-like tyrosine kinase 3 ligand (Flt-3L, PeproTech).

On day 5, equal amounts of bone marrow cells in 3ml of BM culture medium were seeded onto RetroNectin™ (TaKaRa Bio Inc, Shiga, Japan) coated 6-well plate culture dishes which were preloaded with 1ml of retroviral supernatant with 4μg/ml polybrene (Sigma-Aldrich). To enhance co-localisation of virus and cells, bone marrow donor cells were then transduced by additional rounds of spinoculation at 2500×g for 90min with freshly harvested retroviral supernatant every 8h for over a period of 48h. After each spinoculation, the cells were returned to the incubator for continuing culture at 37°C.

On day 7, about $3\text{-}4\times10^6$ cells were used for each transplantation by intravenously injection into the tail vein of 6-8 weeks old recipient mice, which were pre-conditioned with a lethal dose of radiation of 1000 RADS (in a split dose of 600 and 400 RADS within 24h). After 6 weeks the recipient mice were sacrificed and analysed.

2.2.35 FACS-Analysis

Routinely, 10000-50000 cells from each sample were analysed by flow cytometry in a fluorescent activated cell sorter (FACS Calibur, BD Biosciences). Initially by gating based on forward and right-angle light scatter characteristics to determine cell size and granularity. Then cells were simply counted for transduction efficiency calculations and mean fluorescent intensity (MFI) of the eGFP positive cell fraction or gated for fluorescence (eGFP) and re-analysed based on the expression of lineage-specific cell surface markers (myeloid/lymphoid).

2.2.35.1 FACS-Analysis of bone marrow and spleen cells

To determine overall engraftment, myeloid nature and lineage specificity of eGFP positive cells from bone marrow (and spleen) cells suspension were analysed by flow cytometry.

At first, red blood cells were lysed by adding 2.5mL lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, and 0.1mM ethylenediamine tetraacetic acid); then the remaining leukocytes were washed in phosphate-buffered saline containing 2% fetal bovine serum, before

cells were analysed for eGFP fluorescent directly and/or stained with the following antibodies available from BD Pharmingen: CD11b, Gr-1, F4/80, kappaB, CD3ε.

The CD11b antibody identifies myeloid cells, which express the CD11b receptor mainly present on granulocytes, macrophages, dendritic cells and NK cells. The Gr-1 antibody labels myeloid cells with the Gr-1 receptor expressed on neutrophils, granulocytes, monocytes. While the F4/80 antibody is a murine macrophage-specific membrane marker to identify macrophage cells derived from BM precursors and blood monocytes, which is not expressed by lymphocytes. Whereas the CD3ε antibody was employed to label early T cells and kappaB to visualise B- lymphocytes cells.

2.2.35.2 FACS-Analysis: Staining of cells for flow cytometry

1×10^5 to 1×10^6 cells were pre-incubated with 10% rat serum (in staining buffer) for 10-30min, washed twice with staining buffer and incubated for 30-45min on ice with first layer monoclonal antibodies. When appropriate, cells were washed twice between 30-45min incubations with second and third layer antibody-conjugates. Cells were washed twice in staining buffer, once with PBS-Azide and resuspended in 200µl 1% paraformaldehyde in PBS-Azide. Cells were stored at 4°C until analysis within 7 days by flow cytometry using CellQuest software (Becton Dickinson). Cells were stained with iso-type-matched antibodies labelled with PE and the gates were set to exclude 99% of these cells.

Transgene expression in cell populations was analysed by gating based on forward and right-angle light scatter characteristics to determine cell size and granularity. The cells were then gated for fluorescence (eGFP) and re-analysed based on expression of lineage-specific cell surface markers (myeloid/lymphoid).

2.2.36 Colony Assay

Bone marrow cells following retroviral transduction or harvested from the long bones of the recipient mice were plated (2×10^5 or 5×10^5 cells per dish) in 0.66% agar containing α-MEM, 20% fetal calf serum, 2mM-glutamine, 0.2% Na-bicarbonate and

penicillin/streptomycin. The agar was supplemented with the cytokines mIL-1α (10ng/ml), mIL-3 (40ng/ml), rhM-CSF (10ng/ml) and mSCF (200ng/ml). The cell suspension in agar was overlaid on a 1% agar/α-MEM base. Plates were incubated in a humidified incubator at 5% CO_2 for 14 days for colonies to develop before analysis for eGFP fluorescence and phagocyte superoxide activity by NBT assay.

2.2.37 NBT Assay: Phagocyte superoxide assay of bone marrow colonies

For evaluation of the level of reconstitution and function of the transduced gene in the correct cell context we performed the quantitative nitrobluetetrazolium test (NBT) on colonies to assay the activity of the NADPH oxidase complex (Richardson et al., 1998; Wolf et al., 1995). In this test the yellow substrate is converted (reduced) into a purple blue formazan precipitate in cells which express functional NADPH oxidase upon PMA induction.

To assay colonies the cultures were overlaid with a 0.2% NBT solution in PBS containing phorbol myristate acetate (PMA, 1mg/ml). The cultures were incubated at 37°C, 5% CO_2 for 60min to allow the precipitate to form before the numbers of positive / negative colonies were scored under an inverted microscope.

2.2.38 DHR Assay: Phagocyte superoxide assay of peripheral blood

To quantify the leukocyte oxidative burst activity of monocytes and granulocytes in heparinised whole blood the 'Phagoburst Assay Kit' (Orpegen Pharma)(Hirt *et al.*, 1994) was employed, which is highly sensitive (Vowells *et al.*, 1995).

First, the experimental mice were bled by cardiac puncture before termination. Then, using flow cytometry this dihydrorhodamine (DHR) assay determines both the percentage of phagocytic cells and their oxidase activity following PMA induction by detecting reactive oxidants through the conversion of dihydro123 (DHR123) to rhodamine123 (R123).

Neutrophils were identified based on forward and side scatter characteristics. A gate was established to include neutrophils and exclude lymphocytes while at least 10000

events were collected in this gate for all studies. Analysis of neutrophil DHR fluorescence was performed by constructing a side scatter FL2 (DHR) dot plot. Whilst the DHR fluorescence has a wide range of emission spectra (excitation max 505nm, emission max 534nm) that can be measured by either FL1 or FL2 photomultiplier tubes, we excluded the possible interference of the eGFP emission (excitation max 489nm, emission max 508nm) collected in the FL1 detector from the cells of interest (transduced with the bicistronic $p47^{phox}$ cDNA IRES eGFP expression vectors).

DHR-positive cells were identified by gating based on negative (untreated $p47^{phox-/-}$) and positive wild-type control samples. *In vivo* correction of the neutrophil NADPH oxidase system was evaluated in comparison to a standard curve from mixed $p47^{phox-/-}$ and wild-type bloods, e.g. ratios of 0:100%, 20:80%, 50:50% and 100:0%, $p47^{phox-/-}$ to wild-type blood were mixed and used to generate a standard curve of oxidase activity.

2.2.39 Western Blotting: Cell Lysis

Spleens and bone marrow cells were removed from the reconstituted mice and an aliquot of approximately 5×10^6 cells were washed with PBS and lysed as follows: 200µl NP40 lysis buffer (Sigma) were added to the cell pellet, vortexed and left on ice for 10min. Lysates were cleared by centrifugation at 14000×g and 4°C for 5min. The supernatant (200µl) was transferred to a fresh tube and mixed with 200µl 2× SDS sample buffer, boiled for 5min at 100°C before return on ice or stored in the freezer at -70°C.

2.2.39.1 Western Blotting: SDS-PAGE preparation

Flawless glass plates were wiped clean with 70% ethanol. Spacers were placed at each side of one of the glass plates as well as a bottom spacer and locked together. The second glass plate was placed on top and the spacer sponges were pushed to the edge of the smaller plate and clamped together with binder clamps. The gel was prepared by mixing acrylamide, buffer, water, TEMED, and APS together in a universal tube using the appropriate concentrations, e.g. for a 10% gel, 6ml of 30% acrylamide was diluted

with 5ml of gel buffer, 8.8ml H_20, before addition of 20µl TEMED and 200µl 10% APS. Once the APS was added, the SDS-PAGE gel was poured immediately into the top corner of the assembled and upright orientated glass plates until the gel covered ¾ of the small glass plate and was overlaid with water. Once the gel had polymerised, the water was removed and the 4% stacking gel (made of 1.3ml of 30% acrylamide diluted in 2.5ml gel buffer and 6.1ml water before adding 10µl TEMED and 100µl 10% APS) was poured onto the main gel and a comb was inserted and left to polymerise. Once polymerised, the binder clamps were removed and the gel assembly was placed into the gel tank (Hoefer, Harvard Bioscience). Approximately 500ml running buffer was poured into the top and bottom chamber, respectively.

The samples and marker (full range rainbow marker RPN800, 150µg/ml protein, Amersham Life Science) were boiled at 100°C for 5min, quick centrifuged and loaded onto the gel. Aliquotes of 10µl SDS sample buffer lysate, equivalent to 3×10^5 cells were applied to a 10% polyacrylamide gel with a 4.5% stacking gel. The samples and marker were separated by electrophoresis across 100-200V for 1h at 20°C until the running dye front reached the bottom of the gel.

Then the protein was transferred to nitrocellulose membrane by western blotting for immunodetection.

2.2.39.2 Western Blotting: Protein transfer to nitrocellulose filters

Western blotting was performed essentially as described by (Gilmour et al., 2000) using an anti-$p47^{phox}$ antibody (Prigmore et al., 1995) (a kind gift from Prof. Anthony W. Segal, Department of Medicine at the Rayne Institute, University College London). Proteins were electro-blotted onto a supported nitrocellulose membrane (Schleicher & Schuell, London, UK) using the Bio-Rad semi-dry blotter according to the manufacturer's instructions.

First, about 6 pieces of Whatmann 3MM paper and one piece of nitrocellulose membrane the same size as the area of the gel to be transferred was cut out and soaked in transfer buffer for 5min. Then the transfer stack was assembled in the following order: 2 pieces Whatmann, gel, nitrocellulose, 2 pieces of Whatmann. A 10ml plastic

pipette was used to remove any air bubbles between each layer by rolling, before the protein transfer to the membrane-supported nitro-cellulose filter (BDH) was carried out in the BIORAD semi-dry blotter at 12V for 20-30min.

2.2.39.3 Western Blotting: Immunodetection

The membrane was blocked in 5% milk powder-PBS with 0.05% Tween 20 (blocking buffer) for 1h at room temperature or overnight at 4°C before immunodetection with antibodies (3ml blocking buffer with 6µl of $p47^{phox}$ protein primary antibody) for 2-3h at RT. Membrane was washed 5 times with PBS-T before incubation for 30-45min in blocking buffer with 1:1000 anti-rabbit HRP secondary antibody. Then the membrane was again washed five times with PBS-T before incubation for 1min with enhanced chemilluminescence reagent (ECL, Amersham Life Science) and exposed to autoradiography film (KODAK Biomax) for 0.5-5min.

2.2.39.4 Western Blotting: Stripping of nitrocellulose membranes

For verification of the integrity and equal loading of the protein lysates, an anti-β-actin antibody (Sigma) was used as a control.

First, blots were stripped in pre-heated Stripping Buffer (β-mercaptoethanol/SDS/Tris) at 50°C, the western blot added and incubated at 50°C for 30min and then washed three times in PBS-T before being subjected to immunodetection as described above using 3µl of anti-β-actin antibody (Sigma).

Detection was performed by enhance chemiluminescence (ECL, Amersham Pharmacia Biotech, GB) to a horseradish peroxidase conjugated anti-rabbit (Sigma, GB) and anti-mouse antibody (Dako, Cambridge, UK), respectively.

Primary antibody	Dilution	Horseradish peroxidase (HRP)-conjugated second layer antibody	dilution
p47	1:2000	Rabbit anti-goat IgG, 0.4mg/ml (Santa Cruz Biotechnology)	1:500
Mouse anti-β-actin monoclonal IgG, 2.2mg/l (Sigma)	1:1000	Rat antimouse IgG, heavy chain, 1.0mg/ml (Serotec)	1:1000

2.2.40 Statistical Analysis

All luciferase data are represented as mean ±standard deviation (±SD) from four independent transductions or transfections, while statistical analysis was performed by the Mann-Whitney U test; $p < 0.05$. For the distribution of myeloid markers in EGFP positive cells in contrast to total bone marrow cells the Wilcoxon signed-rank test was employed. Both methods are non-parametric tests (distribution-free) which are used to compare two independent groups of sampled data. The P-value associated with this statistic was found from an appropriate table.

3 Cellular promoters for gene expression in myeloid cells

3.1 Background

Genetic modification of pluripotent haematopoietic stem cells (HSCs) offers the possibility of permanent treatment of several inherited and acquired disorders (Dunbar, 1996). In particular, gene transfer into human hematopoietic stem cells with expression targeted to the maturing myelomonocytic progeny has applications for gene therapy of genetic diseases affecting granulocytes and macrophages. Due to the combination of accessibility of stem cells and heterogeneity of tissue locations, the myeloid cell is potentially important as a carrier of therapeutic agents, which is of particular relevance to inherited immunodeficiencies, like our model system – chronic granulomatous disorder (CGD).

In the last few years methods based on viral vectors have been developed for transfer of therapeutic or marker genes into these cells conventionally under control of strong constitutively active regulator sequences, e.g. the viral LTR or exogenous promoters such as the immediate early (IE) promoter of the cytomegalovirus (CMV). However, exclusive expression of the inserted gene in specifically affected lineages, rather than in all progeny of the HSCs, desirable for treating certain genetic disorders more appropriately at clinically relevant levels, has not been shown. Though, retroviral mediated gene expression in human myelomonocytic cells from haematopoietic cell promoters, like CD11b has been investigated in HL60 (Malik et al., 1995), these vectors essentially retained the complete LTR sequences.

Preliminary reports from transient transfections of cell lines have shown that promoters from myeloid- and monocytic-specific genes are relatively more active in myelomonocytic cell lines than in other cell types (Hickstein et al., 1992; Pahl et al., 1992; Shelley and Arnaout, 1991). However, few studies have compared the relative strength of these promoters in retroviral vectors with that of conventionally used viral promoters for applications in gene therapy. Hence, promoters of myeloid-specific

genes that are upregulated with myelomonocytic differentiation may also upregulate expression of an exogenous gene in a retroviral vector (Malik et al., 1995). Therefore, our aim was to identify a myeloid promoter, which has sustained and durable expression in the myeloid lineages and would function successfully in a retroviral construct without interference from a viral LTR.

Myeloid-specific gene expression has been an extensive field of research and the transcriptional mechanisms which regulate myelopoiesis have been the subject of a number of reviews (Clarke and Gordon, 1998; Rosmarin et al., 2005; Skalnik, 2002).

Our initial plan was to investigate a number of characterised *bona fide* myeloid promoters and assess their suitability for incorporation into a retroviral vector by assessment of their ability to direct myeloid-specific transcription of a reporter gene following transient transfection into various myeloid and non-myeloid cell lines.

3.2 Cloning strategy of promoter constructs based on pGL3-Basic

To test for tissue specificity we constructed a series of vectors containing either the luciferase reporter gene (derived form the pGL3 series, Promega) and/or the enhanced green fluorescent protein (eGFP) marker gene (derived form the pEGFP-N1, Clontech) under the control of either myeloid-specific promoters: *human c-fgr* (Kefalas *et al.*, 1995), *human c-fes* (Heydemann et al., 1996; Heydemann et al., 1997), *human CD11b* (Hickstein et al., 1992; Pahl et al., 1992), *human* $p47^{phox}$ (Li et al., 1997; Thrasher et al., 1993), *murine neutrophil elastase* (Nuchprayoon *et al.*, 1994) and *murine PU.1* (Chen *et al.*, 1995a) or non-specific promoters: *SV40*, *CMV* and *human Elongation Factor 1*α (Mizushima and Nagata, 1990).

The pGL3 Luciferase Reporter Vector system (Promega) was selected to provide the basis for the initial quantitative analysis of the promoters available to us. The pGL3 Vectors are improved and redesigned versions of the earlier pGL2 Luciferase Vector System that provide increased levels of expression, due to a modified coding region for firefly (*Photinus pyralis*) luciferase, which had been optimized for monitoring transcriptional activity in transfected eukaryotic cells. As a result, the pGL3 reporter

vectors showed significantly higher expression levels than those obtained with the former pGL2 reporter vectors, while maintaining relatively low background luciferase expression, hence the pGL3 vector system was chosen to provide significantly higher sensitivity, while the assay of this genetic reporter is rapid and quantitative.

Cloning strategies and construction of promoter constructs based on pGL3 luciferase expression plasmid are all illustrated in the Appendix, (paragraph 8.1 and 8.2) however, they are presented here briefly:

The pGL3-Basic vector (see plasmid map 8.2.1) lacks eukaryotic promoter and enhancer sequences, allowing flexibility in cloning putative regulatory sequences. Expression of luciferase activity in cells transfected with this plasmid depend on insertion and proper orientation of a functional promoter upstream of *luc*+. Hence if employed alone it serves as negative control and is used to calculate the background levels of luciferase activity.

The following paragraphs introduce the cellular promoters available to us and cloning strategies of pGL3 reporter constructs.

3.2.1 Myeloid specific gene promoters

3.2.1.1 Murine neutrophil elastase

The neutrophil elastase (NE) gene has been shown to be specifically expressed in immature myeloid cells (Fouret et al., 1989). The NE is a microbicidal protein, a serine protease present in the primary granules of monocytes, granulocytes and is stored in the azurophilic granules of mature neutrophils with functions in tissue turnover and host defence mechanism (Takahashi et al., 1988a). The DNA sequence of the murine neutrophil elastase (mNE) has been found to be significantly homologous to the human neutrophil elastase (hNE) gene (Srikanth and Rado, 1994). Within an about 100bp of the 5'-flanking region of the mNE and hNE gene promoter sequences several functional elements have been identified, including binding sites for PU.1, C/EBP, c-Myb (Oelgeschlager et al., 1996), and PEBP2/CBF in the murine but not the human

gene (Nuchprayoon et al., 1994). Transcription is initiated from a single 30bp sequence downstream from a TATAAA-homologous site (Takahashi et al., 1988b).

For our investigation, an approximately 2400bp DNA fragment of the murine neutrophil elastase promoter was obtained by restriction digest from the plasmid vector named "pTZ19Rclone2" (Nuchprayoon *et al.*, 1994) (see plasmid map 8.1.1) with *Hind*III/*Nco*I and inserted into the same restriction sites on the luciferase expression vector pGL3-Basic to create pGL3-2400mNE-luc (see plasmid map 8.2.3). Similar, pGL3-700mNE-luc (see plasmid map 8.2.4) was cloned from an about 700bp size fragment by restriction of pTZ19Rclone2 plasmid DNA via *Bgl*II/*Nco*I, which was inserted into *Bgl*II/*Nco*I sites of pGL3-Basic.

3.2.1.2 Murine PU.1

The myeloid-specific transcription factor PU.1 (SP-1) has been shown to be a critical component in myeloid differentiation as a member of the *Ets* family of transcription factors. PU.1 is expressed predominantly in myeloid cells (granulocytes, monocytes and macrophages) as well as in B-lymphoid cells of the haematopoietic system, but not in T cells (Chen et al., 1995a; Klemsz et al., 1990; Tenen et al., 1997). PU.1 is also present in eosinophils and found at low levels in primitive CD34+ haematopoietic cells (Voso et al., 1994).

PU.1 has been described as a myeloid master regulator, which autoregulates it's own expression in a positive-feedback system that drives myeloid cell development to completion (Chen et al., 1995a). Together with the transcription factor CCAAT/enhancer-binding protein (C/EBPα) PU.1 is essential for myeloid differentiation into the granulocytic lineage (Borregaard et al., 2001; Sturrock et al., 2004). During haematopoiesis, PU.1 transcription is upregulated during early commitment of pluripotent progenitors to the myeloid lineages and is also required for terminal myeloid gene expression and differentiation (Simon *et al.*, 1996).

Important PU.1 binding sites have been found in nearly all myeloid-cell restricted gene promoters and enhancer sequences studied to date, including genes that encode the growth factor receptors, like macrophage colony-stimulating factor (M-CSF),

granulocyte-macrophage colony stimulating factor (GM-CSF), and granulocyte colony stimulating factor (G-CSF) (Hohaus et al., 1995; Reddy et al., 1994; Smith et al., 1996a; Zhang et al., 1994b) and lysosomal enzymes expressed by myeloid cells (Tenen et al., 1997).

The murine and the human PU.1 gene promoters have both been shown to drive tissue-specific reporter gene expression in myeloid cell lines but not in T cells and non-haematopoietic cells. Its functional promoter regions were demonstrated to lie at two sites within the first 100bp of the PU.1 promoter by deletion analysis (Chen et al., 1995a).

For our investigation, we created pGL3-PU.1-luc (see plasmid map 8.2.7) by linearization of pGL3-Basic with a single *Bgl*II digest and ligation of a 334bp PU.1 promoter fragment from an *Bam*HI digest of pmPU.1-334/luc (Chen *et al.*, 1995a), (see plasmid map 8.1.4) into the resulting backbone of pGL3-Basic.

3.2.1.3 Human CD11b

CD11b is the human leukocyte integrin α-subunit, of the CD11b/CD18 heterodimeric surface antigen, which is located on the surface of human granulocytes and monocytes. The expression of CD11b gene, also called the α-chain macrophage-1 antigen (MAC-1), has been shown to be restricted to cells of the myelomonocytic lineage, e.g. granulocytes, macrophages, monocytes and natural killer cells. The highest levels of CD11b have been found in the most mature myeloid cells, whilst CD11b messenger RNA levels depend upon the stage of differentiation. The CD11b protein (α-chain) forms a noncovalent associated heterodimeric structure with the CD18(β)-subunit, which as CD11b/CD18 heterodimeric surface antigen mediates multifaceted adherence reactivity of myeloid cells (Bauer-TR et al., 1994; Hickstein et al., 1989; Hickstein et al., 1992).

A relatively small (1.7kB) 5` flanking sequence of CD11b gene was found to direct high level of transient reporter gene expression in mature myeloid cells, upon induction of differentiation with either 12-O-tetradecaanoylphorbol-13-acetate (TPA) or retinoic acid

(Pahl et al., 1992). Deletion analysis of the CD11b promoter sequence identified two consensus binding sites required for directing high levels of tissue-specific reporter gene expression. These binding sites lie within only 412bp proximal to the transcriptional start site: a GATA motif, for SP1 and a purine-rich region, a binding site for transcription factors of the *ets* family. And in transgenic mice experiments, the 1.7kb CD11b promoter sequence was confirmed to contain the regulatory elements sufficient for myeloid-specific expression in macrophages *in vivo* (Dziennis et al., 1995), while in comparative studies, which compared the CD11b promoter against CD18 and CD34, it was established that the CD11b promoter showed the most favourable results in terms of levels of expression, inducibility with differentiation and lineage specifity (Malik et al., 1995),

For our investigation, the CD11b promoter (Hickstein et al., 1992; Pahl et al., 1992) was obtained as a kind gift from Dr. D. Hickstein in form of the pΦGH-CD11b vector (see plasmid map 8.1.7).

First, we created pGL3-1672CD11b-luc (see plasmid map 8.2.8) taking the 1672bp *Bam*HI(blunted)/*Hind*III fragment which was inserted into the *Hind*III/*Nco*I(blunted) sites of pGL3-Basic. The resulting 1672bp sized CD11b-luciferase vector was then truncated by 464bp through *Bgl*II digest and religation to pGL3-1272CD11b-luc (see plasmid map 8.2.9), while pGL3-732CD11b-luc (see plasmid map 8.2.10) resulted from 982bp deletion by *Sst*I restriction and religation of the pGL3-1672CD11b-luc plasmid.

3.2.1.4 Human c-fgr

The human *c-fgr* gene is a proto-oncogene, which belongs to the *src* family of non-receptor protein tyrosine kinases (Link *et al.*, 1992). It is one of the major myelomonocytic promoters, which is active during differentiation of myeloid cells, with the maximum expression being detected in the terminal stage of differentiation. Dominant sites of *fgr* mRNA expression are myelomonocytic cells and tissue macrophages, e.g. *fgr* protein has been found to accumulate in human HL-60 cells when these were induced towards monocytic or granulocytic differentiation (Miyazaki et al., 1993).

Previous studies of the *c-fgr* promoter have shown that - with respect to the transcriptional start site - the region from nucleotide -344 to -116 is required for basal activity of the *c-fgr* promoter in U937 cells, and that the 5`-region from nucleotide -1211 to -752 is responsive to PMA (Kefalas et al., 1995).

For our investigation, the 1762bp DNA sequence of the human *c-fgr* promoter was re-cloned by *Nco*I (blunted)/*Hind*III digest from pGL-3145 ((Kefalas *et al.*, 1995) a pGL2-derivate, see plasmid map 8.1.2) and inserted into pGL3-Basic in the *Sma*I/*Hind*III site to create pGL3-1762cfgr-luc (see plasmid map 8.2.5). Similar a 1231bp *Sma*I/*Sma*I fragment of the *c-fgr* promoter was ligated into the *Sma*I site of pGL3-Basic to clone pGL3-1231cfgr-luc (see plasmid map 8.2.6).

3.2.1.5 Human c-fes

The protein product of the *c-fes* (*c-fps/fes*) proto-oncogene encodes a non-receptor tyrosine-specific cytoplasmic protein kinase of 92kDa. It has been implicated in the normal development of haematopoietic tissues (immature and differentiated myeloid cells, e.g. macrophages, neutrophils and granulocytes), to be involved in haematopoietic growth factor signalling and also to be expressed in vascular endothelial cells in adult mammals (Heydemann et al., 1996; Heydemann et al., 1997; Heydemann et al., 2000).

Previously, a 13kb human genomic construct of the *c-fes* gene was shown to direct lineage restricted and integration site independent expression in transgenic mice (Greer et al., 1990), though transient-transfection experiments demonstrated that plasmids containing only 446 base pairs of *c-fes* promoter linked to a luciferase reporter gene directed exclusive expression in myeloid cells, whilst the functional binding sites for Sp1, PU.1, and a novel nuclear factor FEF (*c-fes* expression factor) required for myeloid cell-specific expression were shown to be located within 151 base pairs of *c-fes* 5'-flanking sequences (Heydemann et al., 1996). No other DNA element within the 13kb human *c-fes* locus were found to contain any other positive *cis*-acting elements, with the exception of a weakly active region within the 3'-flanking sequences (Heydemann et al., 1996).

The *c-fes* promoter was not available for our initial investigation; hence cloning strategy and reporter vectors are described in the following chapter.

3.2.1.6 Human p47phox

As described, $p47^{phox}$ is one of the essential components of the phagocyte NADPH oxidase (*phox*). Studies of the $p47^{phox}$ promoter have shown that less than 500bp make up the essential sequence of the $p47^{phox}$ promoter (Li et al., 1997; Li et al., 1999; Marden et al., 2003a; Marden et al., 2003b) and that an essential PU.1 consensus binding sequence (GAGGAA) is located on the non-coding strand from position -40 to -45 relative to the transcriptional start site of the $p47^{phox}$ gene (Li et al., 1999; Marden et al., 2003b). While a promoter construct extending to -46 was sufficient to drive tissue-specific expression of the luciferase reporter gene, reporter expression was significantly increased with the extension of the promoter from -46 to -48 (Li et al., 1999). The binding avidity of the transcription factor PU.1 to these sequences is closely correlated with the capacity to dictate reporter gene transcription. Point mutations introduced into the PU.1 binding consensus sequence at position -46 (G) and/or position -47 (T) reduced both reporter expression and PU.1 binding, whereas a point mutation at position -48 had no effect (Li et al., 1999). The importance of the functional PU.1 binding site was also confirmed in parallel studies on the promoter sequence of the CD18 integrin gene. Mutations introduced for nucleotides G and T at positions -76 and -77 (which are the corresponding positions -46 and -47 in the $p47^{phox}$ promoter) reduced PU.1 binding and promoter activity (Li et al., 1999).

The $p47^{phox}$ promoter was not available for our initial investigation, hence cloning strategy and reporter vectors will be discussed and described in Chapter 4.

3.2.2 Constitutive Promoters

3.2.2.1 LTR

Most studies with retroviral vectors in the mammalian cells use vectors that drive gene expression from the Moloney murine leukemia virus long-terminal repeat (MLV-LTR). This promoter drives strong gene expression in numerous different cell types, however it also has been found to be silenced in cells possessing stem cell characteristics, such as

embryonic carcinoma cells, embryonic stem cells and haematopoietic stem cells (Gorman et al., 1985; Kempler et al., 1993; Lange and Blankenstein, 1997).

LTR driven vectors and retroviral constructs will be discussed in a later chapter, but the LTR as constitutive promoter is mentioned here shortly to present a complete list of the constitutive promoters used as positive controls in our investigation.

3.2.2.2 SV40

The SV40 early promoter is used in many commercial available expression systems and cloning vectors today. The SV40 promoter originates from the Simian Virus 40 (SV40), a polyomavirus that is found in both monkeys and humans. Like other polyomaviruses, the SV40 is a DNA tumor virus, but commonly persists as a latent infection. The SV40 virus itself is of approximately 5000bp in size and has been extensively investigated since its identification in the 1950's as a model for eukaryotic transcription and replication.

In the pGL3-Control plasmid (Promega, see plasmid map 8.2.2) the SV40 promoter is employed upstream of the luciferase gene and used as a positive control and internal standard for promoter and enhancer activities expressed by pGL3 recombinants, providing strong constitutive expression of the luciferase reporter gene in many types of mammalian cells.

To stay within the plasmid nomenclature of this thesis the pGL3-Control vector was re-named to pGL3-SV40-luc.

3.2.2.3 CMV IE

The cytomegalovirus (CMV) major immediate early (IE) promoter is one of the strongest constitutive promoters employed and is used in many commercial available eukaryotic expression systems and cloning vectors today. The strength of the CMV promoter results from its own enhancer element, which contains an array of imperfect direct repeats which have binding properties to transcription factors like, nuclear factor

kappa-B (NF-κB) and cAMP response element binding protein (CREB) (Sambucetti et al., 1989; Wilkinson and Akrigg, 1992).

For our initial experiments, we created pGL3-CMV/T7-luc (see plasmid map 8.2.11) with the CMV promoter taken from Invitrogen's expression vector pDNA3CAT (Invitrogen, see plasmid map 8.1.8) from a digest with *Bgl*II/*Hind*III, which was inserted into the same restriction sites of pGL3-Basic backbone plasmid. A second CMV luciferase expression vector was created from pATCMVIXCAT3 (see 8.1.9) carrying an approximately 850bp *Xho*I/*Hind*III fragment with the CMV immediate early (CMV IE) promoter (with an IntronX sequence at the 3'end) which was inserted *Xho*I/*Hind*III into pGL3-Basic to create pGL3-850CMV-luc (see plasmid map 8.2.13).

3.2.2.4 Elongation factor 1α (EF1α)

The promoter of the translation elongation factor 1 alpha (EF1α) gene was identified to drive powerful mammalian expression from a plasmid vector (Mizushima and Nagata, 1990). EF1α is one of the most abundant proteins in eukaryotic cells and a counter part of E.coli EF-Tu, which promotes the GTP-dependent binding of aminoacyl-tRNA to ribosomes. EF1α is expressed constitutive in almost all kinds of mammalian cells and its promoter was previously used for reconstitution of $gp91^{phox}$ in (Zhen *et al.*, 1993).

For our vectors, we made use of a 1189bp fragment of the human EF1α flanked by *Eco*RI/*Hind*III taken from pEF-PGK-Neo ((Mizushima and Nagata, 1990; Zhen et al., 1993), see plasmid map 8.1.5) and ligated it into the *Nco*I(blunted)/*Hind*III sites of the pGL3-Basic cloning vector, which resulted to in the plasmid pGL3-1189EF1alpha-luc (see plasmid map 8.2.14).

These constructs were then tested for reporter gene expression by transient assays.

3.3 Transient expression of reporter genes driven by cellular promoters

Myeloid cell lines such as PLB985, HL-60 and U937 provide an excellent model system for studying myeloid differentiation due to their ability to differentiate from their immature state into cells, that morphologically and functionally resemble mature granulocytes and macrophages on exposure to various chemical inducers (Pahl et al., 1991) and consequently, these cell lines are used routinely to study the regulation of differentially expressed genes and their cellular promoters.

For our initial investigation, we performed a series of transient transfections with our pGL3-promoter constructs (as described in 3.2) to assess the transcriptional activity of the luciferase reporter gene under control of various cellular promoter at 24 hours post transfection.

3.3.1 Transient Transfection by Electroporation

Since myeloid cell lines are refractory to transfection by calcium phosphate, the transient transfection method by electroporation developed by (Pahl et al., 1991) was employed with the aim to assay for differences in promoter activity driving the luciferase gene in U937 (and HL60 cells, data not shown).

The results shown in Figure 10 and Figure 11 indicated an enormous (over 200-300× fold) quantitative difference between the transient luciferase activities of the constitutive promoters compared to the activity from the myeloid cell promoter constructs in U937 cells. While, the luciferase activity from the myeloid constructs in untreated U937 was found to be too low for any comparisons between the various myeloid promoter constructs, the addition of 1ng/ml phorbol myristate acetate (PMA) had an enhancement effect on the reporter gene activity in induced U937 cells. The largest increase in luciferase activity was observed with the CMV promoter constructs in induced U937 cells, while EF1α appeared to be less or not effected, and the SV40 promoter activity was insignificantly low against the strength of the CMV and EF1α. Among the myeloid promoters the PU.1 and CD11b promoters performed best followed by the cfgr promoter, and the mNE promoter at background level.

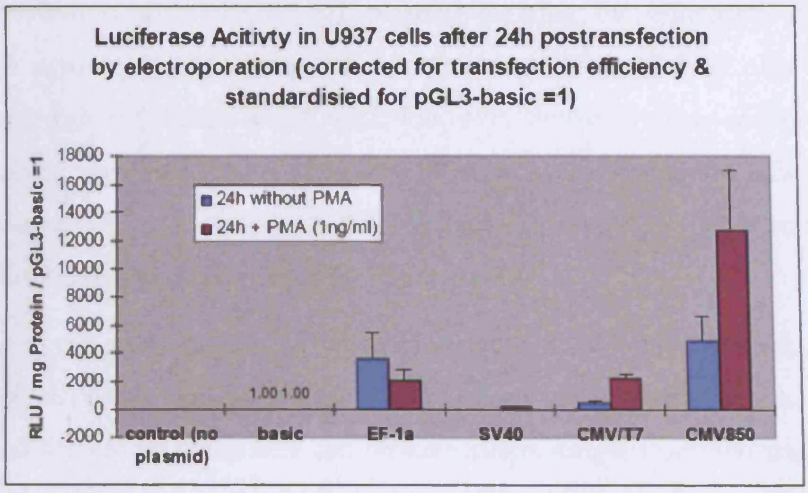


Figure 10: Transient luciferase activity and effect of ±PMA on constitutive promoters in U937 cells 24h posttransfection by electroporation.

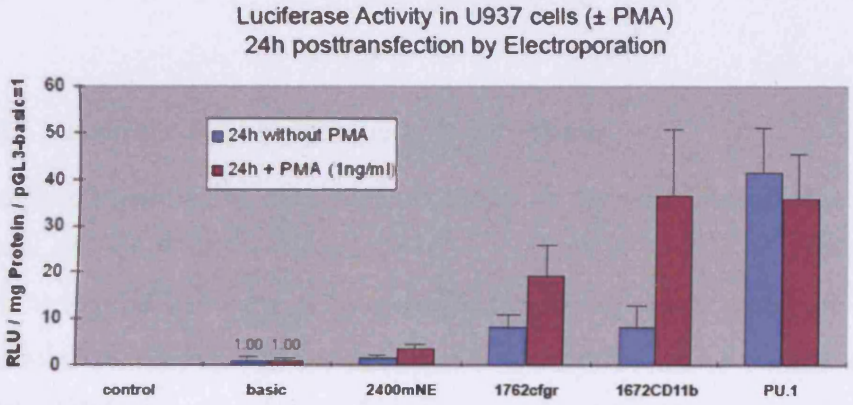


Figure 11: Transient luciferase activity and effect of ±PMA on myeloid promoters in U937 cells 24h posttransfection by electroporation (±PMA).

U937 cells were co-transfected with experimental pGL3-"promoter"-luc and pEGFP-N1 plasmid DNA, as described in the Materials and Methods (2.2.23.1). Half of the cells were induced with 1ng/ml PMA (red bars) and harvested 24h post-transfection. Cell-free supernatants were prepared and assayed for luciferase activity and total protein concentration (Materials and Methods: 2.2.24). Values are corrected for transfection efficiency by the number of EGFP positive cells and standardised for pGL3-basic = 1 to correct the PMA effect on the background activity. Enzyme activity is expressed as relative light units per milligram (RLU/mg). The data are given as mean values of four independent transfections ± standard deviation (±SD). The pGL3-"promoter"-luc constructs are abbreviated as indicated under each column set (Figure 10 with constitutive promoters and Figure 11 with myeloid promoters) including a negative control without plasmid.

Co-transfection with pEGFP-N1 plasmid carrying the enhanced green fluorescent protein reporter gene indicated that transfection efficiencies of only about 4% were achieved with U937 cells, which while low, were similar to those observed by others for cell lines of this type (Pahl et al., 1991). However, electroporation efficiencies for HL60 cells was significant lower at only 0.5-1% (data not shown), which made it impossible to accurately assess luciferase activities in these cells.

Owing to the problems we encountered with electroporation of myeloid cells, e.g. high level of cell death, and poor viability of various myeloid cell lines, as well as the time required for cell recovery and cell differentiation longer than 48h transient expression of luciferase reporter and standardisation of the luciferase assay system, we concluded that transfection by electroporation seemed inappropriate to achieve the goals set out for our project and we turned to a more gentle transfection method with Lipofectin : integrin peptide : DNA (LID) complexes.

3.3.2 Transient Transfection by LID complexes

Though still undergoing continued development the availability of a new transfection method developed by Dr. Steve Hart (Hart et al., 1998; Uduehi et al., 2003) at the time in the Molecular Immunology Unit, enabled us to experiment with a much gentler and more efficient method of transfection. The so-called "LID vector" consist of the plasmid DNA complexed with Lipofectin and a polycationic 16-lysine tail peptide, which is conjugated to an integrin-targeting ligand of about 10 amino acid residues (Hart et al., 1998). This technique has the advantage of a higher transfection efficiency, while measuring reporter gene activity from a much smaller, but totally viable cell population.

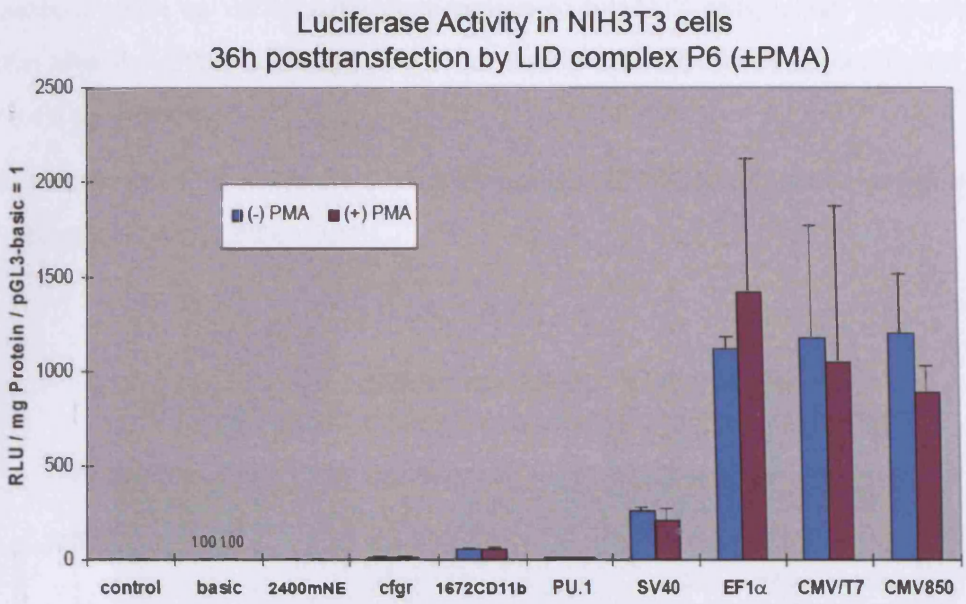


Figure 12: Transient luciferase activity of promoter constructs in NIH3T3 cells 36h post-transfection by LID complex P6 (±PMA)

NIH3T3 cells were co-transfected with experimental pGL3-"promoter"-luc and pEGFP-N1 plasmid DNA, as described in the Materials and Methods (2.2.23.2). Half of the cells were induced with 1ng/ml PMA (red bars) and harvested 36h post-transfection. Cell-free supernatants were prepared and assayed for luciferase activity and total protein concentration (Materials and Methods: 2.2.24). Values are corrected for transfection efficiency by the number of eGFP positive cells and standardised for pGL3-basic = 1 to correct the PMA effect on the background activity. Enzyme activity is expressed as relative light units per milligram (RLU/mg). The data are given as mean values of four independent transfections ± standard deviation (±SD). The pGL3-"promoter"-luc constructs are abbreviated as indicated by the relevant promoter under each column set and a negative control without plasmid.

The tissue selectivity of the myeloid specific constructs is shown in (Figure 12). Luciferase assays were taken from all our promoter constructs 36h posttransfection from fibroblastic NIH3T3 cells, representing a non-myeloid cell line. While transfection efficiency - as determined by the proportion of EGFP-positive cells - was roughly equivalent for all the promoter constructs (about 70-80% in NIH3T3 cells), the luciferase activity of the myeloid constructs (mNE, cfgr, CD11b, and PU.1) was negligible in comparison to the luciferase activity of the constitutive promoter constructs (CMV, SV40 and EF1α), implying that the myeloid promoters functioned poorly in a non-myeloid cell. In contrast, to what was observed in myeloid cells, PMA

had minimal effect on the reporter gene activity in NIH3T3 cells, which is consistent with the idea that PMA mediates its effects through terminal differentiation and not directly on the promoters.

The results obtained for luciferase activity from the constitutive promoter constructs in U937 cells are shown in Figure 13.

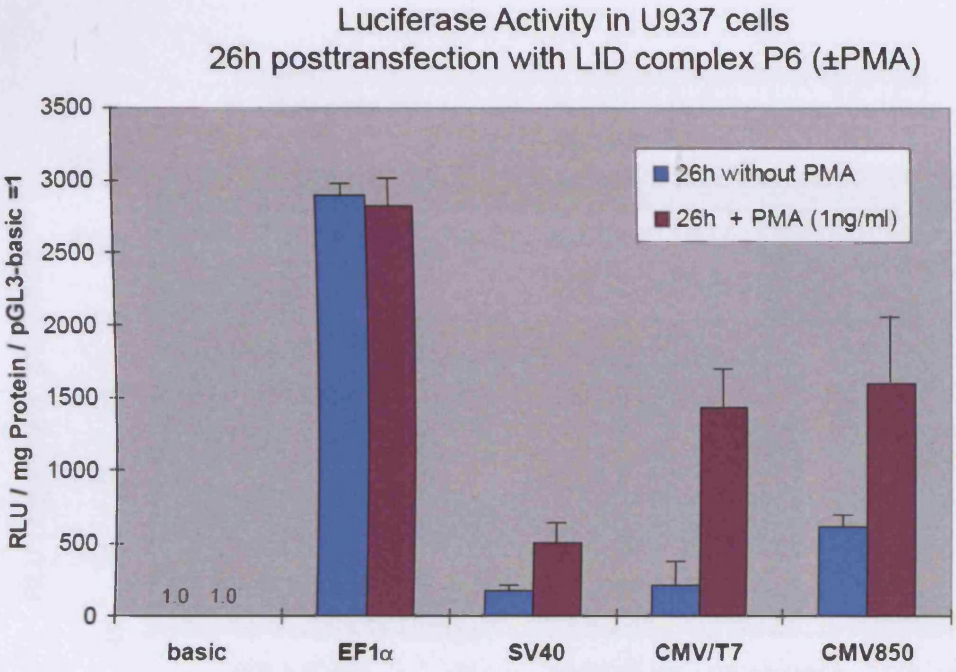


Figure 13: Transient luciferase Activity of constitutive promoter constructs in U937 cells 26h post-transfection by LID complex P6 (±PMA).

U937 cells were co-transfected with experimental pGL3-"promoter"-luc and pEGFP-N1 plasmid DNA, as described in the Materials and Methods (2.2.23.2). Half of the cells were induced with 1ng/ml PMA (red bars) and harvested 26h post-transfection. Cell-free supernatants were prepared and assayed for luciferase activity and total protein concentration (Materials and Methods: 2.2.24). Values are corrected for transfection efficiency by the number of eGFP positive cells and standardised for pGL3-basic = 1 to correct the PMA effect on the background activity. Enzyme activity is expressed as relative light units per milligram (RLU/mg). The data are given as mean values of four independent transfections ± standard deviation (±SD). The pGL3-"promoter"-luc constructs are abbreviated as indicated by the relevant promoter under each column set.

The results obtained for luciferase activity from the myeloid promoter constructs in U937 cells are shown in Figure 14, which includes a set of truncated versions of the

CD11b promoter constructs of 1672bp, 1226bp, and 732bp respectively. Earlier studies on the CD11b promoter sequence had demonstrated that only 412bp proximal to transcriptional start site of the CD11b gene are required to drive tissue-specific reporter gene expression(Pahl et al., 1992), and therefore in foresight to reduce the packaging load in a retrovirus vector construct later, the 1672bp of the CD11b was reduced to 732bp (as described previously in 3.2.1.3).

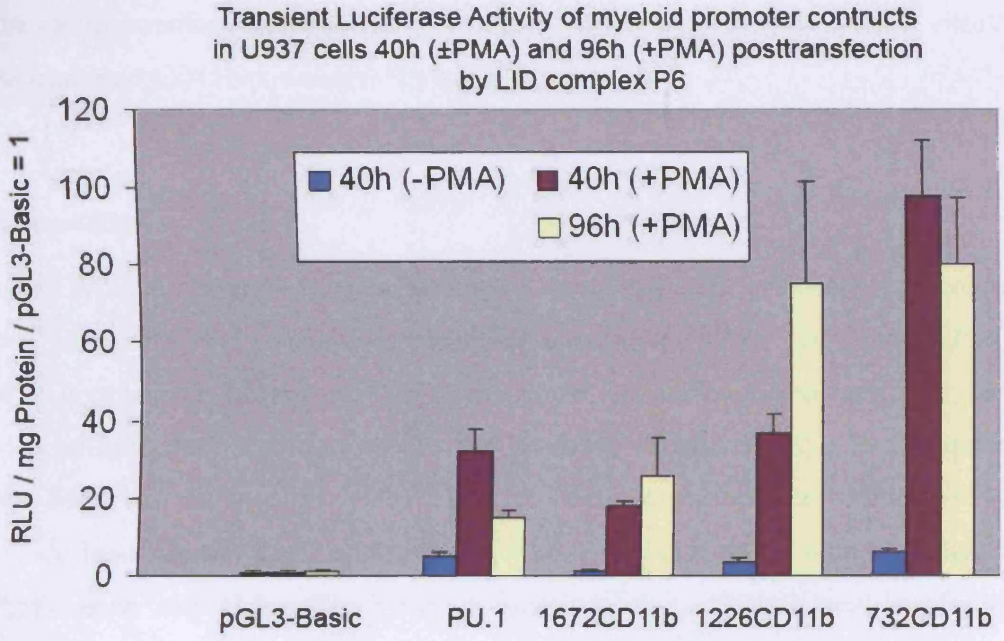


Figure 14: Transient luciferase Activity of PU.1 and truncated versions of CD11b promoter constructs in U937 cells 40h (±PMA) and 96h (+PMA) post-transfection by LID complex P6

U937 cells were co-transfected with experimental pGL3-"promoter"-luc and pEGFP-N1 plasmid DNA, as described in the Materials and Methods (2.2.23.2). Half of the cells were induced with 1ng/ml PMA (red bars) and harvested 40h and 96h post-transfection. Cell-free supernatants were prepared and assayed for luciferase activity and total protein concentration (Materials and Methods: 2.2.24). Values are corrected for transfection efficiency by the number of eGFP positive cells and standardised for pGL3-basic = 1 to correct the PMA effect on the background activity. Enzyme activity is expressed as relative light units per milligram (RLU/mg). The data are given as mean values of four independent transfections ± standard deviation (±SD). The pGL3-"promoter"-luc constructs are abbreviated as indicated by the relevant promoter under each column set.

The transfection efficiency in U937 cells - as determined by the proportion of EGFP positive cells - was about 40%, representing a significant improvement over the transfection efficiency achieved by electroporation previously.

While the myeloid promoter constructs if compaired to the constitutive promoters constructs performed poorly in untreated U937 cells, luciferase activity from truncated CD11b reporter and the PU.1 promoter constructs showed inducibility when U937 cells were subjected to induction by PMA 40h and 96h posttransfection. Kinetics of the PMA-induced transcriptional enhancement appeared to be similar to those obtained from the electroporation transfections. The induction effect by PMA was most visible with the truncated CD11b constructs (Figure 14).

3.4 Discussion

Despite the fact that the reporter gene activity of myeloid-specific promoters constructs was low relative to the constitutive promoter constructs (SV40, CMV and EF1α) myeloid-cell promoter activity in U937 cells could be demonstrated, and this was enhanced by induction of cells with phorbol myristate acetate (PMA). In particular, both the *human CD11b* and the *murine PU.1* promoter constructs were transcribed at significantly higher levels than the activity of *human c-fgr* and *murine neutrophile elastase.* Indeed, the *murine neutrophile elastase* promoter construct was particularly weak displaying levels barely higher than background, consistent with similar results described for this promoter (Nuchprayoon et al., 1994).

Particularly interesting was the inducibility of the *human CD11b* constructs. The 1672bp CD11b promoter showed only background levels of luciferase activity without PMA, which was similarly poor as obtained with the *c-fgr* promoter in untreated cells (Figure 11), but showed potent induction of up to 4× fold higher levels of expression after PMA treatment of U937 cells, and this was evident with either transfection methods employed.

In particular, the truncated version carrying only 732bp of the *CD11b* minimal promoter sequence showed itself to be best in terms of inducibility. These results were consistent with previous reports showing that endogenous *CD11b* expression levels

were low or undetectable in myeloid precursors, and steadily increased during myeloid differentiation, reaching the highest levels in mature neutrophils, monocytes, and macrophages (Dziennis et al., 1995).

In contrast, the level of expression of the *murine PU.1* promoter construct, which in untreated cells was 5 fold stronger than *CD11b*, proved to be less sensitive to PMA induction, approximately 5-6× induction. These expression patterns would be consistent with the fact that *in vivo* PU.1 functions exclusively in a cell intrinsic manner to control the development of granulocytes, macrophages, and B lymphocytes. Thus *PU.1* is upregulated during early commitment of multipotential progenitors and controls myelopoiesis by regulation of both proliferation and differentiation pathways (Tenen et al., 1997).

Two different technologies were employed to introduce DNA into cell lines, which are quite resistant to transfection. Although each method has advantages and disadvantages, they required compromise on one or the other aspect of the experimental set-up and reproducibility.

Firstly, electroporation is the most versatile method of DNA transfection because it has been shown to work on a wide variety of cell types. This technique relies on high-voltage pulses that cause the cell membrane to break down, producing transient pores through which DNA can enter. By varying the electric field strength, and length of time the cells are exposed to the electric field, it is possible to optimise electroporation parameters for essentially any cell type, which represents the main advantage of this technique. However, this method also results in high cell mortality and necessitates greater quantities of DNA than liposome-mediated techniques.

In contrast, lipofectamine-mediated transfection is based on a mixture of a polycationic synthetic lipid and a neutral fusogenic lipid with an amine-group, which results in the formation of unilamellar liposome vesicles that have a net positive charge due to highly-positive amine headed groups on these molecules, which spontaneously form lipid-DNA complexes due to the ionic interaction to neutralize the negatively charged phosphate groups on the DNA (Felgner et al., 1994; Felgner et al., 1987; Radler et al.,

1997). These structures can then fuse and pass through the plasma membrane to deliver DNA into cells, a process thought to be improved upon by incorporation of integrin targeting peptides (Hart et al., 1997; Hart et al., 1998; Uduehi et al., 2003). Consequently, this gene delivery technique is less harmful to the cells than electroporation, but may be limited by endosomal degradation and is variable depending on the cell type, and efforts were made in the design of different integrin peptide sequences to partially overcome this problem (Hart et al., 1998).

Nonetheless, the results of transient transfection experiments in general are probably not indicative of any effects on the regulation of the endogenous gene from the induced expression of a myeloid promoter. Nor are they necessarily a prediction of stable expression patterns, due to the lack of selectable markers.

Although some information was obtained that CD11b might be a candidate, it needed further testing in a stable transduced cell environment and in a retroviral backbone integrated into a host cell genome to establish its suitability for the use in gene therapy. In fact, previous results in our laboratory had shown that transient assay results are not always a reliable predictor of activity of stably integrated constructs (Marden et al., 2003a; Marden et al., 2003b).

In conclusion, in order to investigate the suitability of the one or other myeloid promoter in a stably integrated host cell environment the following Chapter will focus on retroviral constructs utilising the *CD11b* and *PU.1* promoter and the assay of their ability to drive reporter gene expression in transduced myeloid cell lines, before and after induced differentiation.

4 Reporter Gene Expression in Cell lines transduced with retroviral vectors containing cellular promoters

4.1 Background

The use of retroviral vectors driving expression of an inserted "therapeutic gene" from the viral LTR suffers from a number of problems. *Firstly*, these are often subject to inconsistent levels and duration of expression due to their natural propensity to acquire *de novo* methylation *in vivo* (Challita et al., 1995; Challita and Kohn, 1994). *Secondly*, the levels of transcription may be highly sub-optimal in specific cell types; previous work in our own laboratory had suggested that this may have contributed to a relatively low level of oxidase expression in CGD-patient B cells lines corrected by transduction with such a retroviral vector (Thrasher et al., 1992). *Thirdly*, the random nature of retroviral integration makes the transduced cells susceptible to insertional mutagenesis and as indicated earlier, a significant hazard from this derives from the influence of the retroviral long terminal repeat (LTR), which as well as commonly providing the vector with a promoter function, is also a potent transcriptional enhancer (Coffin et al., 1997). Because the enhancer is functional in a broad range of tissue types, genes adjacent to the site of retroviral integration are susceptible to major up-regulation of their level of transcription. This could have major consequences if one of these is an oncogene, which was brought sharply into relief recently, in the course of otherwise highly successful clinical trials (Cavazzana-Calvo et al., 2000a; Hacein-Bey-Abina et al., 2002a; Ott et al., 2006; Thrasher et al., 2006).

One strategy to improve tissue or cell-specific transgene expression has been the use of vectors driving expression from promoters endogenously active in a particular lineage. However, it was found that the viral enhancer-promoter located in the 5'LTR interferes with the cis regulatory elements inserted in the vectors, resulting in unstable proviral transmission and/or poor transgene expression (Emerman and Temin, 1984; Emerman

and Temin, 1986a; Emerman and Temin, 1986b; Persons and Nienhuis, 2000; Proudfoot, 1986).

More recently, self-inactivating (SIN) retroviral vectors that lack enhancer/promoter sequences carrying a deletion in the U3 region of the LTR have been used to produce vectors lacking the LTR enhancer sequences (Iwakuma et al., 1999; Yu et al., 1986), which are thought to activate adjacent genes, and to drive transgene expression from internal cis-regulatory sequences. With this methodology it should be possible to prevent the competition that has been observed between inserted promoters and the LTR (Akgun et al., 1991; MacKenzie et al., 1994; Schambach et al., 2006), and may also reduce the risk of insertional mutagenesis and help to circumvent the *in vivo* silencing of gene expression that has been observed with viral promoters (Challita and Kohn, 1994).

Consequently, we designed our vectors with a deleted U3 region of the LTR. Because the 3`LTR is duplicated during the retroviral life cycle and copied to the 5`end of the vector it is only necessary to delete the 3`LTR to produce a vector, which lacks endogenous regulatory elements in the provirus (as described previously, 1.2.5). Thus, it is possible to delete enhancer sequences from the 3`LTR whilst still utilising an intact 5`LTR to direct transcription in retroviral producer cells. In the target cells, the integrated provirus obtains its 5`LTR sequences from the 3`LTR carrying the enhancer deletion, and so the integrated vector will lack any viral-derived enhancer sequences (Δ-LTR vector).

4.2 Tissue-specific promoters in viral vectors

Previous attempts have been made to produce retroviral vectors with a tissue-specific transcriptional tropism, this has been of particular relevance to gene therapy approaches to treating cancer, where it was crucial to restrict transgene expression specifically to the tumour (Jager et al., 1999; Malik et al., 1995; Mavria et al., 2000; Miller and Vile, 1995; Vile et al., 1995).

Retroviral vectors directed at therapy of monogenic inherited conditions have generally accepted the wide tropism of viral LTR for expression of inserted genes, though a few

recombinant viral vectors have been described which have incorporated tissue-specific enhancer elements in attempts to limit or optimise transcriptional activity to the particular cell-type affected by the disease.

One such area has been in gene therapy for the thalassaemias where obtaining high level erythroid lineage expression is desired. Vectors incorporating globin gene promoter elements to drive the human β-globin gene have been constructed but, though erythroid-specific, these tended to suffer from low and variable levels of expression (Sadelain et al., 1995). Subsequent studies have established that retroviral vectors could be markedly improved by the incorporation of sequences from the, normally distant, locus control region (LCR). The LCR is a chromatin region that functions to regulate tissue specific and temporal activity of the entire β-like globin gene cluster. By incorporating core site vectors, the HS2, HS3 and HS4 core sites of the human β-globin LCR into a retroviral vector they acted as a powerful erythroid enhancer, but failed to provide the position-independent expression associated with the activity of the endogenous LCR (Sadelain et al., 1995; Sadelain et al., 2000).

Despite patchy results, some notable successes have been reported in generating vectors with tumour specific expression patterns. Vectors employing the murine tyrosinase gene promoter to drive cytokine genes, or the HSVtk suicide gene expression in melanoma cells (Vile et al., 1994a; Vile et al., 1994b; Vile and Hart, 1993a; Vile and Hart, 1993b). However, partial loss of specific gene expression from these vectors was observed due to promoter interference by the strong promoter/enhancer elements in the U3 region of the viral LTR and also by an additional internal viral SV40 promoter which directed marker gene expression (Vile et al., 1994a). This led to the development of alternative vector design where the LTR enhancer and/or promoter sequences were interchanged with corresponding minimal cellular regulatory elements from the human tyrosinase promoter. Resulting vectors demonstrated expression of GM-CSF in tissue-specific manner, however at relatively low levels of expression (Diaz et al., 1998).

Another example, the endothelial-specific prepro-endothelin-1 (ppET-1) promoter (Mavria et al., 2000) has been employed to limit expression to endothelial cells. In these

constructs the ppET-1 promoter sequences were inserted into a U3-deletion region in the enhancer of the MFG vector, rather than in the body of the construct. In a tumour model, therapeutic levels of expression from this hybrid ppET-1/LTR enhancer were obtained (Mavria et al., 2005).

Two major attempts have been made to investigate the use of myeloid-cell-specific promoter elements in retroviral vector constructs. The first of these made use of promoters from the β-integrin genes CD11a, CD11b and CD18 (Bauer-TR et al., 1994). When tested in HL60 cells these promoters were only capable of directing low levels of transcription, which showed only marginal increases on induction of myeloid differentiation with PMA or retinoic acid. However, these constructs also contained fully functional LTRs, so the levels of transcription may have been seriously affected by competition between the different promoters.

In another similar study, the use of the CD11b, CD18 and CD34 promoters was compared with the activities of viral promoters CMV and SV40 in transduced HL60 cells. In this instance, some evidence for tissue specificity and up-regulation of gene expression on induction of myeloid differentiation was obtained. In particular, the CD11b promoter showed as much as an 8 fold increase in transcription, when the HL60 cells were differentiated to granulocytes with retinoic acid.

It is not instantly clear why the two studies differed so significantly but the precise regions of the promoter used appeared to be different, with the latter study surprisingly only using a 404bp proximal region. Also, the second study assayed transcription by levels of glucocerebrosidase (GC) activity, through inclusion of a GC cDNA in the vector rather than adenosine deaminase, as in the study by (Bauer-TR et al., 1994). Determining GC activity was confounded to some degree by the presence of endogenous GC activity in the HL60 cells, which may have influenced the findings, though in general these were corroborated by Northern blotting.

In the following section we describe our results obtained using LTR enhancer-deleted and non-deleted retroviral vector constructs. These retroviral vectors used the most

promising of the myeloid specific-gene promoter elements, assayed earlier, to drive reporter gene expression in a number of myeloid and non-myeloid cell lines.

4.3 Cloning strategy and retroviral constructs based on pMBΔ

Our initial investigations were based on the availability of the enhancer-deleted retroviral vector pMBΔ (see plasmid map 8.3.1), a kind gift from Dr. C. Porter, Chester Beatty Laboratories, London. The pMBΔ retroviral vector is derived from the pBabe series of MLV retroviral vectors developed by (Morgenstern and Land, 1990). While the pMBΔ 5'LTR from position 1-1375 and 1965-4125 is from the original pBabe vector, the DNA sequence from position 1375-1965 carrying the 3'LTR enhancer deletion, originated from eukaryotic expression vector pMPSV developed from the myeloproliferative sarcoma virus (Artelt et al., 1988).

Cloning strategies and construction of the retroviral vectors with the pMBΔ backbone are illustrated in detail in the Appendix (paragraph 8.3), however a brief description is presented here: Due to the fact that the pMBΔ plasmids multiple cloning site (MCS) was limited to only three unique sites, which are *Bam*HI, *Cla*I and *Eco*RI and the luciferase gene in pGL3-Basic could only be taken out by a *Cla*I digest, which essentially is the entire coding region of the luciferase reporter gene including its polyA signal, our cloning strategy was to insert all promoter-luciferase reporter units in anti-sense orientation to the 5'LTR of pMBΔ, in order to avoid premature termination of the retroviral RNA transcripts during retroviral packaging.

Further we anticipated that with this cloning strategy, transcription of the reporter gene in target cells would occur exclusively from the exogenous promoters we were investigating, while also excluding any possible background levels of remaining LTR activity, though the enhancer deletion of the provirus.

First the retroviral backbone pMBΔ was linearised with a *Cla*I digest and dephosphorylated to avoid religation, before inserting the luciferase reporter gene from pGL3-Basic (see plasmid map 8.2.1) as a *Cla*I/*Cla*I fragment. The orientation of the insert was then tested by *Xba*I digest to verify the creation of pMBΔ-Basic-luc (see

plasmid map 8.3.2). This construct was designed to act as a negative control vector, without an exogenous promoter driving the luciferase reporter.

4.3.1 Retroviral pMBΔ constructs with myeloid specific promoters

pMBΔ-2400mNE-luc (see plasmid map 8.3.4) was cloned by inserting the approximately 2400bp sequence of the mNE promoter and luciferase reporter from pGL3-2400mNE-luc (see plasmid map 8.2.3) via the same *Cla*I/*Cla*I cloning strategy, while for the creation of pMBΔ-700mNE-luc (see plasmid map 8.3.3) it was possible to extract the truncated 700bp long mNE promoter with the luciferase reporter gene by *Bgl*II/*Bam*HI digest from pGL3-2400mNE-luc, which was inserted into the *Bam*HI unique cloning site of pMBΔ. The orientation of the insert was tested again by *Xba*I digest.

However, based on the weak expression data obtained previously (Figure 11), the mNE vectors were not investigated.

The pMBΔ-PU.1-luc (see plasmid map 8.3.5) vector was created by inserting the PU.1-luciferase sequence unit from pGL3-PU.1-luc (see plasmid map 8.2.7) as *Cla*I/*Cla*I fragment into *Cla*I linearised pMBΔ backbone. A *Xba*I digest was again used to verify the orientation of the insert.

pMBΔ-1672CD11b-luc (see plasmid map 8.3.6) was made by *Cla*I digest of pGL3-1672CD11b-luc digest and ligation of the expression unit into the *Cla*I site of pMBΔ. Again, the orientation of the insert was tested by *Xba*I digest.

4.3.2 Retroviral pMBΔ constructs with constitutive promoters

The SV40-luc insert of pMBΔ-SV40-luc (see plasmid map 8.3.7) was taken via *Cla*I digest from pGL3-Control (see plasmid map 8.2.2) and orientation was tested by *Xba*I digest. As additional positive controls pMBΔ-CMV/T7-luc (see plasmid map 8.3.8) was

created by cloning the CMV/T7-luc insert from pGL3-CMV/T7-luc (see plasmid map 8.2.11) via *Bgl*II/*Bam*HI digest into the *Bam*HI site of pMBΔ. While pMBΔ-850CMV-luc (see plasmid map 8.3.9) was cloned using the *Cla*I sites from pGL3-850CMV-luc (see plasmid map 8.2.13) and pMBΔ.

Finally, pMBΔ-1189EF1α was created by *Cla*I digest of pGL3-1189EF1α-luc (see plasmid map 8.2.14) and ligation into the *Cla*I site of pMBΔ. As before the orientation of the insert subject of verification by *Xba*I restriction.

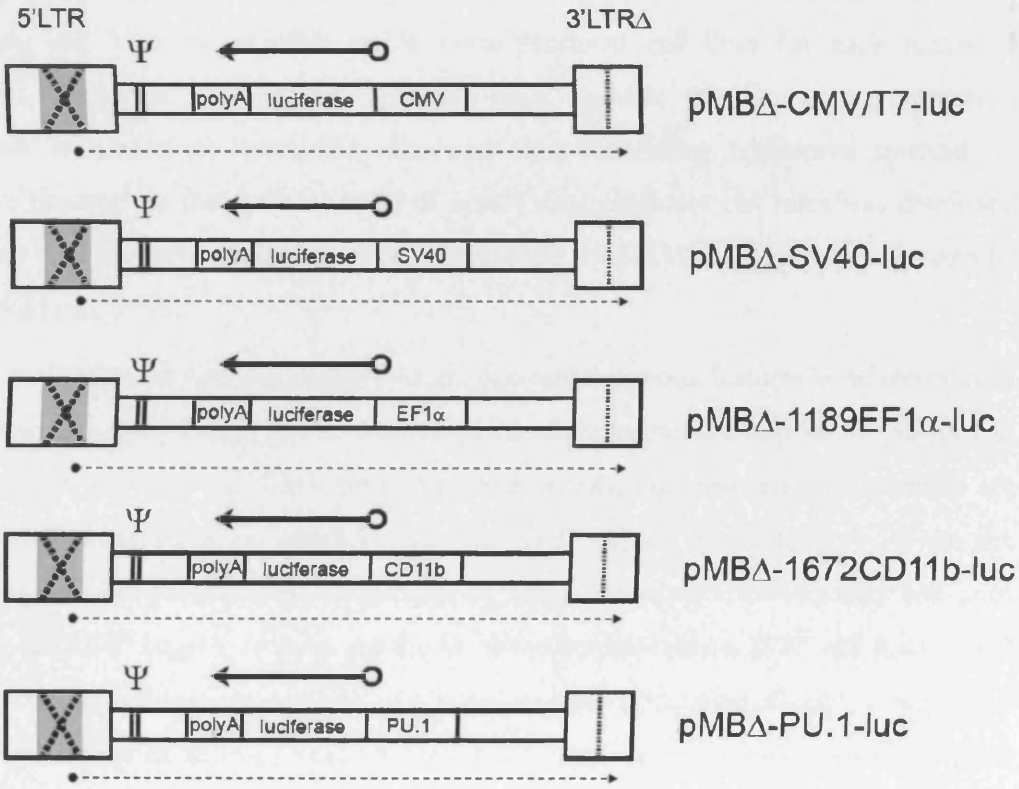


Figure 15: Schematic diagrams of the retroviral vectors based on pMBΔ

The expanded box at each end represents the LTR in which the grey area identifies the 75-bp enhancer repeat region. The enhancer deletion in the 3`LTR is indicated by the dotted line and the cross in the 5`LTR indicates that the deleted LTR will be copied and transposed to the 5`end in the provirus. Ψ indicates the location of the packaging signal. The arrows above each vector schematics indicates the luciferase reporter gene transcription unit in antisense

(dotted arrows) to the LTR driven transcription of genomic viral RNA. Detailed plasmid maps are listed in the Appendix 8.3.

Although the entire set of described pMBΔ-promoter-luciferase vectors were created, virus was initially produced only from the constitutive constructs pMBΔ-CMV/T7-luc and pMBΔ-SV40-luc as controls and pMBΔ-1672CD11b-luc and pMBΔ-PU.1-luc to investigate myeloid specificity (Figure 15).

4.3.3 Packaging and Retroviral Transduction with pMBΔ based vectors

Initially it was planned to use AM12 (Markowitz et al., 1988) or FLY (Cosset et al., 1995) packaging cell lines to establish stable virus producer cell lines for each retroviral construct. However, due to the relatively high number of the various retroviral promoter constructs at hand, the otherwise time-consuming traditional method of selective cloning for the establishment of stable viral producer cell lines was dismissed in favour of a transient packaging system developed for MLV-based retroviral vectors by (Soneoka et al., 1995).

In this three-plasmid viral packaging system, two advantageous features were introduced to achieve high titre: *Firstly*, the in *trans* supplied viral structural components *gag-pol* and *env* are expressed from the CMV promoter, and *secondly*, the structural components are placed on separate plasmids which contain the SV40 origins of replication. When the three plasmid components are introduced by transient co-transfection into cell lines carrying the SV40 large T antigen, e.g. highly transfectable human 293T cell lines – and in the presence of sodium butyrate to further increase expression of each component - this combination of features were reported to enhance retroviral gene expression, and to obtain helper-free viral stocks of approximately 10^7 infectious units/ml within 48 h after transient co-transfection (Soneoka et al., 1995).

In our hands however, sufficient titres could not be reproduced with our recombinant pMBΔ vectors. Although transfection efficiency via Calcium Phosphate precipitation was optimised using the pHIT111 plasmid (Soneoka et al., 1995) carrying the lacZ

reporter, the viral titre achieved were insufficient even to infect NIH3T3 and HeLa cells. A disadvantage of the system seemed to be the inability to select for the retroviral structural genes and the necessity for simultaneous transfection of the three different plasmid for a sufficient titre. However, transient production systems have the potential to yield high-titre supernatant and do not require the lengthy process of identifying a high-titre producer cell clones typical of most stable packaging cell line systems (Yang et al., 1999).

Fortunately, with the availability of a second generation retroviral producer cell line (Kinsella and Nolan, 1996), we were able to avoid the technical difficulties we had with the transient three-plasmid system described above. The Phoenix™ producer cell lines are based on a human 293T cell line, which is highly transfectable with either calcium phosphate-mediated or lipid-based transfection protocols and in which the nuclear replication and retention functions of the Epstein-Barr virus (EBV) are utilised to maintain retroviral constructs episomally. Due to the *gag-pol* episome in this cell line can be amplified with hygromycin selection, while the envelope protein episomes carry a diphtheria toxin resistance gene as a selectable marker.

4.3.4 Luciferase activity obtained with pMBΔ constructs

In the first set of transduction experiments, we compared the performance of the Phoenix Ampho and Phoenix Eco packaging system with our pMBΔ constructs.

Given that the Phoenix™ producer cell lines provide the viral packaging components *gag-pol* and *env* (eco or ampho) constitutively in *trans*, we only had to perform a single plasmid transfection and were able to obtain measureable luciferase activity from our constitutive promoter constructs in a preliminary comparison of the luciferase activity in transduced NIH3T3 cells with virus derived from both packaging cell lines, Phoenix™ Ampho and Eco (Figure 16).

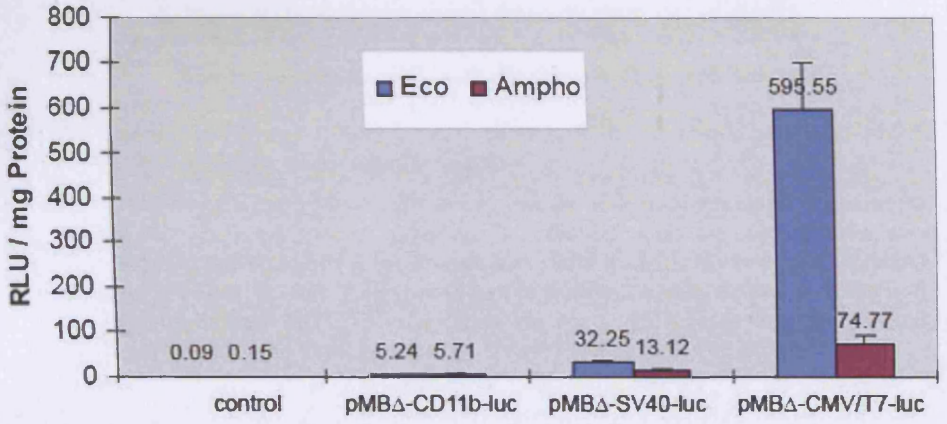


Figure 16: Luciferase gene activity in NIH3T3 cells transduced with pMBΔ retroviruses derived from Phoenix Ampho and Phoenix Eco packaging cell lines.

NIH3T3 cells were subjected to a single round of transduction with same volume of supernatant containing the experimental pMBΔ retroviral vectors derived from Phoenix Ampho and Phoenix Eco producer cell lines as indicated. However, it was not possible to control for any differences in titre that may have resulted from the use of different promoter constructs. Cell-free supernatants were prepared and assayed for luciferase activity and protein concentration (Materials and Methods: 2.2.24). Background (control) activity was measured by assay from non-transduced NIH3T3 cells. Data are mean ±SD from four independent transductions and luciferase activity is expressed as RLU/mg protein.

The improved level of luciferase activity obtained with virus derived from the Phoenix™ Eco producer was not unexpected and this proved to be beneficial to our *in vivo* experiments using $p47^{phox-/-}$ knockout mice planned later on (described in detail in the following chapter). By subjecting NIH3T3 cells to additional cycles of transduction with the amphotropic vectors, a three fold increase in luciferase activity from our pMBΔ-CMV/T7-luc vector was achieved as shown below (Figure 17).

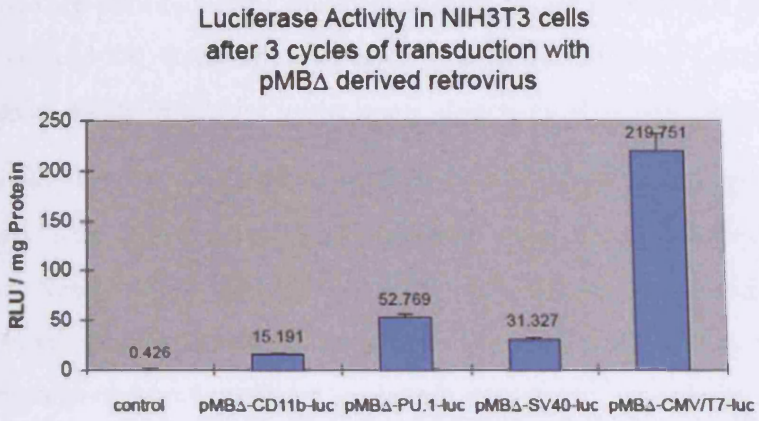


Figure 17: Luciferase activity in NIH3T3 cells after 3 cycles of transduction with pMBΔ vector packaged with Phoenix Ampho.

NIH3T3 cells were subjected to three rounds of transduction with experimental pMBΔ retroviral vectors (ampho) as indicated. Cell-free supernatants were prepared and assayed for luciferase activity and protein concentration (Materials and Methods: 2.2.24). Background (control) activity was measured by assay from non-transduced NIH3T3 cells. Data are mean ±SD from four independent transductions and luciferase activity is expressed as RLU/mg protein

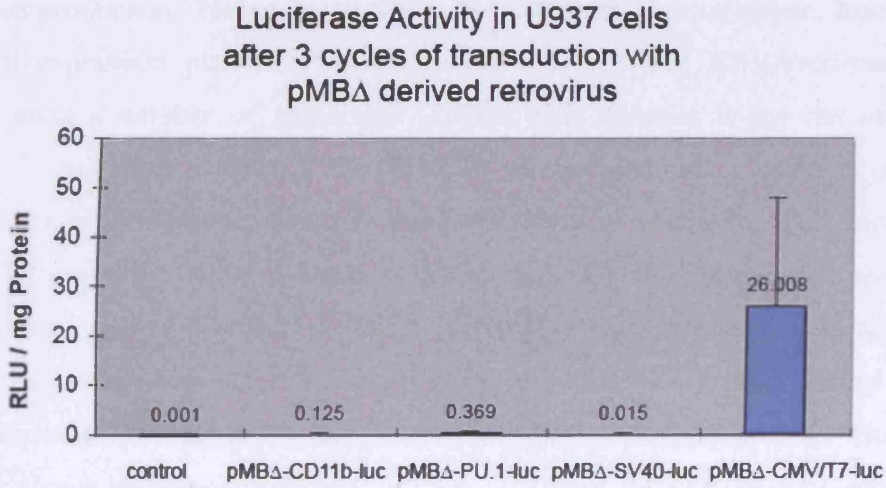


Figure 18: Luciferase activity in U937 cells after 3 cycles of transduction and spinocculation with pMBΔ vector

U937 cells were subjected to three rounds of transduction with experimental pMBΔ retroviral vectors (ampho) as indicated. Cell-free supernatants were prepared and assayed for luciferase activity and protein concentration (Materials and Methods: 2.2.24). Background (control) activity was measured by assay from non-transduced U937 cells. Data are mean ±SD from four independent transductions and luciferase activity is expressed as RLU/mg protein

However, when we transduced the myelomonocytic cell line U937 with pMBΔ-derived viral vector (Figure 18), even after repeated rounds of infection by spinocculation, luciferase activity was poor relative to the levels obtained with NIH3T3 cells (Figure 17).

Despite the fact that we were able to transduce myeloid and non-myeloid cells with pMBΔ vectors after repeated cycles of infection using transient packaging cell line (Kinsella and Nolan, 1996), luciferase activity obtained from transduced cells were significantly lower than expected. Although low levels of transduction to myeloid cell lines had been reported previously, we suspected that poor titres may be a consequence of the anti-sense-orientation of the promoter-luciferase transcript in our viral construct in the pMBΔ vectors, which we had not anticipated in the context of the 293T-based packaging cell line, particularly with the myeloid specific promoter driving the transgene.

However, a more detailed analysis of the titres from pMBΔ vectors was not carried out due to the availability of an alternative retroviral vector at the time with enhanced features and compatibility to the Phoenix Ampho and Eco packaging cell lines for high-titre virus production. Hence, we turned to this alternative vector system, based on the retroviral expression plasmid PINCO (Figure 19), a hybrid EBV/retroviral vector incorporating a number of important features, that optimise it for the use in the Phoenix^{TM} packaging system: The PINCO vector harbours a different retroviral backbone to pMBΔ vector, it carries the EBV origin of replication and encodes the Epstein-Barr Virus Nuclear Antigen (EBNA) facilitating its maintenance as a stable episome and it can be selected for using a puromycin resistance marker. In addition, it carries the green fluorescent protein (GFP) reporter gene under control of the immediate early CMV promoter, for *in vivo* evaluation of transduced cells. The system has been shown to generate viral titres as high as 10^7 colony forming units/ml and was successfully tested for high-efficiency gene transfer to human haematopoietic progenitor cells (Grignani et al., 1998).

While the PINCO vector carries the EGFP reporter gene under the control of the same immediate early CMV promoter sequence as the luciferase reporter gene in our pMBΔ-CMV/T7-luc construct, PINCO-CMV-luc (see plasmid map 8.5.6) was fast created by

exchanging the EGFP gene of PINCO through a *Hind*III/*Not*I digest with the luciferase reporter gene *Hind*III/*Xma*III from pGL3-Basic (Promega, see plasmid map 8.2.1) and tested in NIH3T3 and U937 cells for transduced luciferase activity that demonstrated the superiority of the PINCO vector (data not shown).

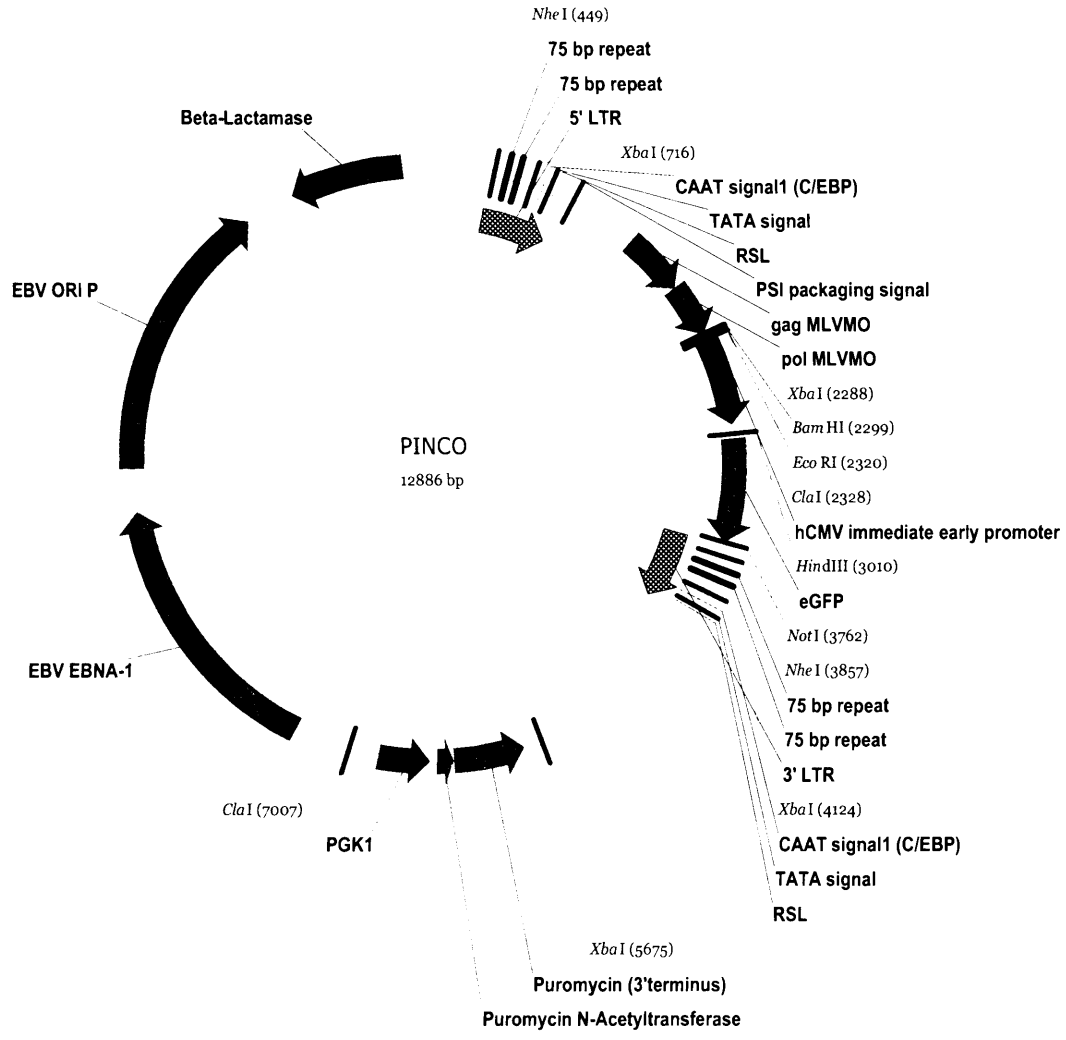
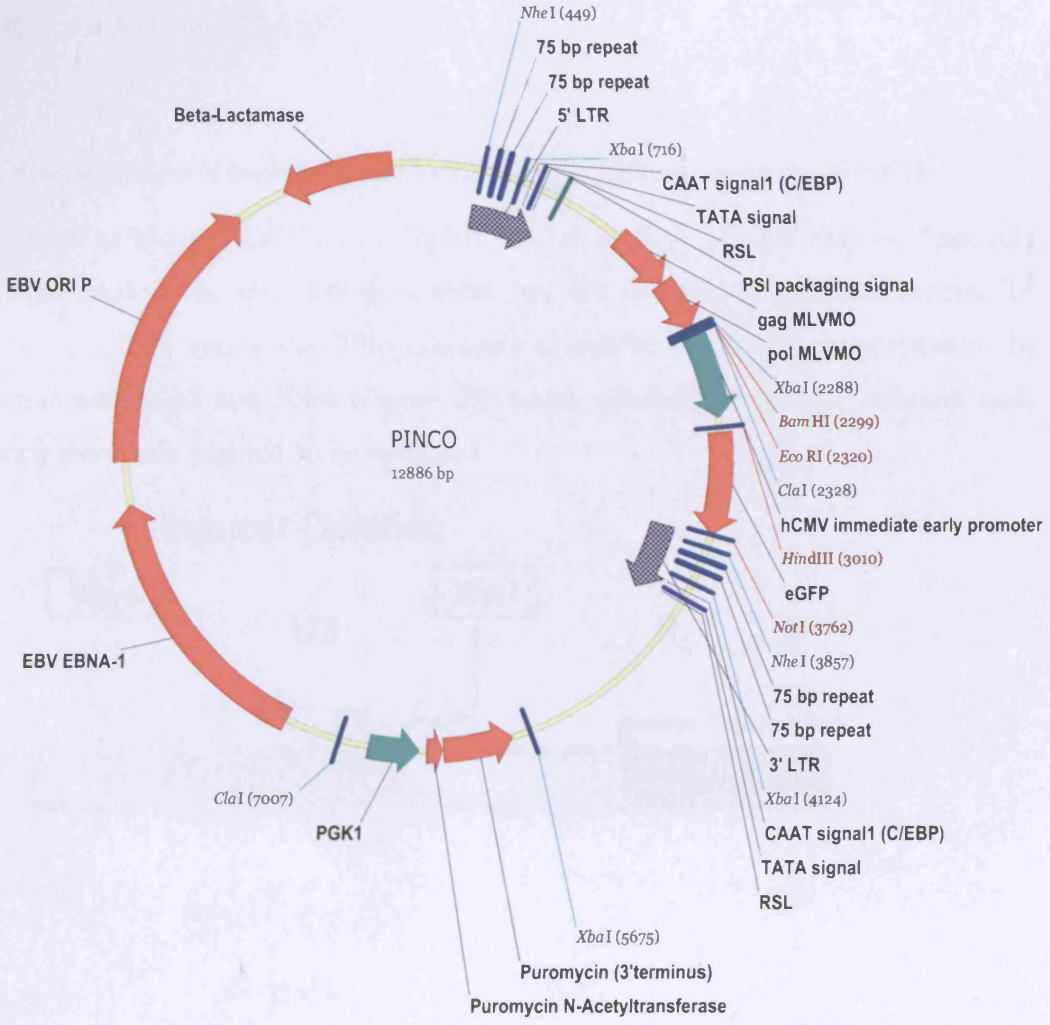


Figure 19: Schematic representation of the retroviral vector PINCO.

Limited restriction map (unique restriction sites in red) and features of the PINCO vector: the EBV origin of replication and the EBNA-1 gene, which facilitates replication as an episome; the puromycin resistance gene (selectable marker) under control of the PGK-1 promoter; the full-length Moloney LTRs, extended ψ sequence, and the EGFP reporter gene under control of the exogenous CMV IE promoter. The 75-bp repeats (enhancer), TATA, C/EBP and the R region stem-loop (RSL) signals are identified in the LTRs, and the *cis*-

exchanging the EGFP gene of PINCO through a *Hind*III/*Not*I digest with the luciferase reporter gene *Hind*III/*Xma*III from pGL3-Basic (Promega, see plasmid map 8.2.1) and tested in NIH3T3 and U937 cells for transduced luciferase activity that demonstrated the superiority of the PINCO vector (data not shown).



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acting encapsidation sequence (PSI, ψ) located between 5`LTR and the *gag* gene. Sequence data from (Grignani et al., 1998), personal communication.

Consequently, we decided to created a completely new set of constructs based on the PINCO retroviral backbone (Grignani et al., 1998), which were optimised for the use with the Phoenix™ packaging system (Nolan laboratories, Stanford, USA, see also Materials and Methods, 2.1.5.3).

4.4 Construction of enhancer-deleted (Δ-LTR) vectors based on PINCO

The vector backbone PINCO (see Figure 19 and detailed plasmid map in Appendix 8.5.1) was used as the basis for the construction of our enhancer-deleted vectors. To generate a Δ-LTR vector the 75bp enhancer repeats in the 3`LTR were removed by digestion with *Nhe*I and *Xba*I (Figure 20) which produce compatible cohesive ends allowing the vector plasmid to be re-ligated.

Figure 20: Schematic representation of retroviral LTR and enhancer deletion

Detail showing the enhancer deletion between *Nhe*I and *Xba*I. The deletion removes both of the 75-bp repeat sequences of the enhancer, which contains a number of transcription factor binding sites, but leaves the promoter intact. (adapted from (Coffin et al., 1997). The binding proteins that recognise the enhancer repeats are indicated: Positive-acting transcriptional control elements are represented by multiple members of the ETS family of transcription factors bind to two sites in each 75bp repeat unit (Gunther and Graves, 1994). The

core-binding factor (CBF) is a heterodimeric binding protein that recognises the enhancer core region (Ogawa et al., 1993a; Ogawa et al., 1993b; Wang et al., 1993). Genetic and biochemical evidence suggests that Ets and CBF proteins cooperate to activate transcription mediated by the enhancer (Sun et al., 1995; Wotton et al., 1994). Nuclear factor 1 (NF1), belongs to a small family of DNA binding proteins (Reisman, 1990), which binds to two sites in each repeat. Mammalian type C retrovirus enhancer factor 1 (MCREF1) is a DNA binding protein, which overlaps the ETS and CBF binding sites. The glucocorticoid receptor (GR) also binds to the enhancer (DeFranco and Yamamoto, 1986). Glucocortoids have been reported to activate LTR driven reporter genes (Miksicek et al., 1986). The GR binding site overlaps an E box element, which is recognised by several transcription factors from the basic helix loop helix (bHLH) structural family (Cornelissen et al., 1991; Nielsen et al., 1992). Negative acting transcriptional control elements are located upstream of the 75bp repeat, which bind to the embryonic LTR binding factor (ELP), which is a member of the nuclear hormone receptor superfamily (Tsukiyama et al., 1992) and has been shown to have modest repressor activity on the LTR. Another cellular factor, upstream conserved region binding protein (UCRBP; also known as Yin Yang 1 (YY-1) or NF-E1) binds this region and mediates repression of the LTR in transient expression assays (Flanagan et al., 1992).

To create an LTR-driven vector, the CMV promoter was deleted and vectors were also constructed in which the EGFP sequences were replaced by a luciferase reporter gene which could be quantified more accurately.

4.4.1 Cloning strategy and retroviral vectors derived from PINCO

Cloning strategy and construction of the PINCO derived vectors are described in the Appendix, paragraph 8.5, however some are also presented here briefly:

PINCO-Δ: To create PINCO-Δ (abbreviation of PINCO-Δ-CMV-eGFP, enhancer-deleted 3'LTR with CMV promoter, see plasmid map 8.5.5) the following sub-cloning steps were carried out, first, plasmid pGL3-Basic (Promega, plasmid map 8.2.1) was digested with *Nhe*I *Xba*I and religated to remove the *Nhe*I *Xba*I sites, creating pGL3-Δ-NX (see plasmid map 8.5.2). We then took a *Sal*I *Sal*I fragment from the retroviral expression vector PINCO and sub-cloned it into the *Sal*I site of pGL3-Δ-NX to create pGL3-ΔNX-LTR (see plasmid map 8.5.3). Digestion of this construct with *Nhe*I *Xba*I and religation resulted in pGL3-ΔNX-Δ-LTR (see plasmid map 8.5.4), which carried the deletion of the 3'LTR enhancer region between *Nhe*I and *Xba*I. Finally, the

SalI/SalI fragment was returned to PINCO-*SalI/SalI* to create PINCO-Δ (plasmid map 8.5.5).

Δ-*LTR-eGFP:* (abbreviation of PINCO-Δ-Basic-eGFP, enhancer-deleted 3`LTR without CMV promoter, see plasmid map 8.5.8) was created by *NotI/Bam*HI digest of PINCO-Δ. The resulting backbone was ligated with the *BglII/NotI* EGFP fragment of pEGFP-N1 (Clontech, see plasmid map 8.4.1).

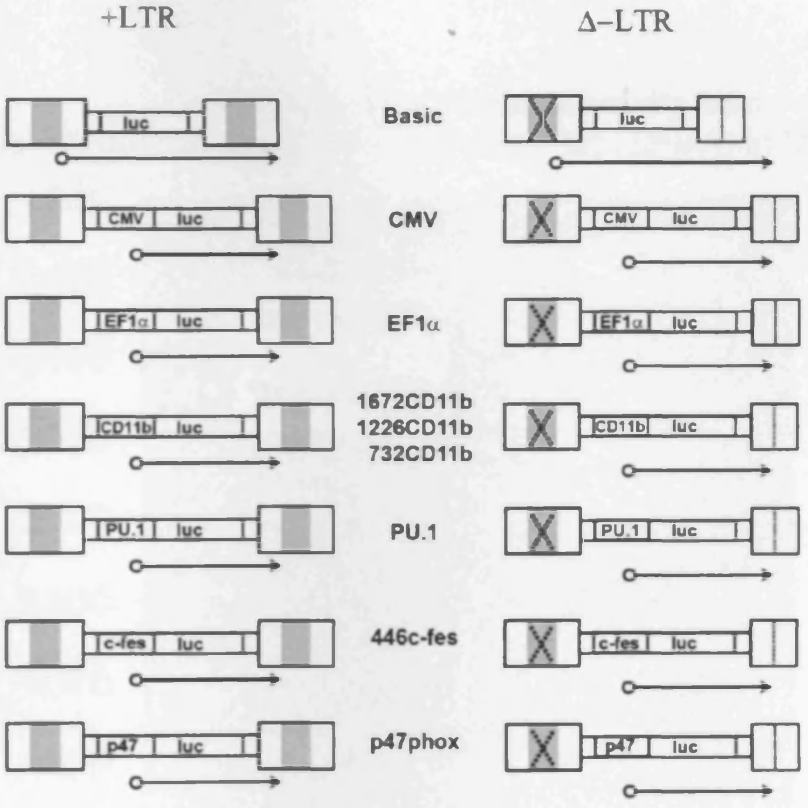


Figure 21: Schematic representation of PINCO derived retroviral vectors and the enhancer-deleted derivates with luciferase (or EGFP) reporter gene expression.

Schematic diagrams of the PINCO vectors with luciferase (or eGFP, not shown) reporter gene under control of various promoters as indicated. Vectors with wild-type LTRs (+LTR) are on the left panel, enhancer-deleted derivates (Δ-LTR) are on the right. The expanded box at each end represents the LTR in which the grey area identifies the 75-bp enhancer repeat region. The enhancer-deletion in the 3`LTR is indicated by the dotted line and the cross in the 5`LTR indicates that the deleted LTR will be copied and transposed to the 5`end in the provirus. The arrows underneath each vector schematics indicates the luciferase reporter gene transcription unit. Detailed plasmid maps are listed in the Appendix 8.5.

Luciferase activity obtained with PINCOΔ-CMV-luc (enhancer-deleted 3`LTR) and PINCO-CMV-luc (intact LTRs) vectors form transduced U937 cells (±PMA) were compared in a preliminary experiment (Figure 22). Whilst PMA induced cell differentiation increased luciferase activity in U937 cell under control of the CMV promoter (without LTR enhancer) about 1.6× fold, luciferase activity obtained from PINCO-CMV-luc was enhanced by 2.4× fold.

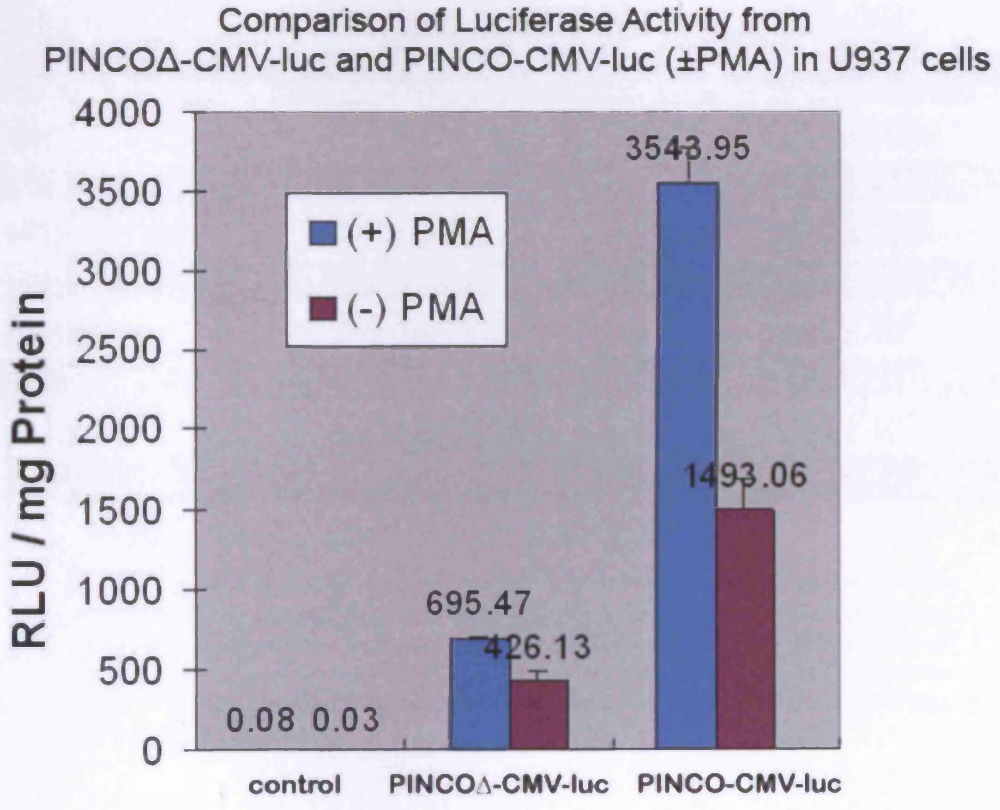


Figure 22: Comparison of Luciferase Activity obtained with PINCOΔ-CMV-luc and PINCO-CMV-luc vector transduced U937 cells (±PMA).

U937 cells were transduced by spinocculation with PINCOΔ-CMV-luc and PINCO-CMV-luc retroviral vectors (ampho) as indicated and induced ±PMA before assayed for luciferase activity as described in the Materials and Methods (2.2.24). Background activity (control) was measured by assay from non-transduced U937 cells. Data are mean ±SD from four independent transductions. Luciferase activity is expressed as RLU/mg protein.

4.4.2 Effects of the LTR enhancer deletion on titre and transcription

To test the effects of the enhancer deletion on titre, the wild-type and Δ-LTR vectors carrying the EGFP reporter were used to transduce 3T3 cells. Retroviral titre, as estimated by the proportion of EGFP-positive cells (Table 3), was not significantly affected by the enhancer deletion.

	Vector Titre (ifu/ml)	
Promoter	**Δ-LTR**	**WT-LTR**
Basic (LTR)	**1.16×10^7**	**1.07×10^7**
CMV	1.12×10^7	1.24×10^7
1189EF1α	**0.94×10^7**	**1.03×10^7**
1672CD11b	1.10×10^7	1.70×10^7
1226CD11b	**0.53×10^7**	**0.78×10^7**
732CD11b	0.64×10^7	0.95×10^7
PU.1	**0.78×10^7**	**1.03×10^7**
p47phox	0.61×10^7	1.49×10^7
446cfes	**1.00×10^7**	**0.90×10^7**

Table 3: Effect of enhancer deletion on retroviral titre: EGFP vectors

The effect of enhancer deletion on retroviral titre is represented by comparison of ifu/ml from Δ-LTR vs WT-LTR constructs with the EGFP reporter gene. Titre was determined using amounts of viral supernatant between 1 and 500 µl and the value ifu/ml calculated from the population of EGFP positive NIH3T3 cells taken in the linear region of the curve. Described in the Materials and Methods 2.2.31.

Following construction, the luciferase vectors were tested for titre by Northern dot blot from retroviral supernatants derived from each construct (Figure 23).

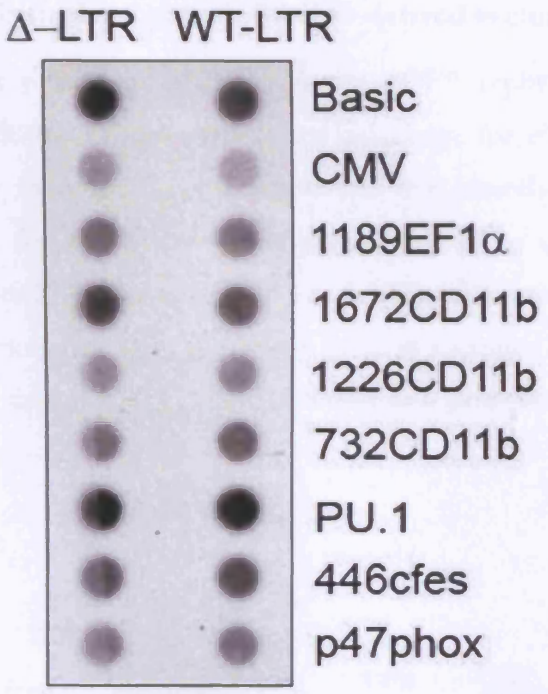


Figure 23: Effect of enhancer deletion on retroviral titre: Luciferase vectors.

The effect of enhancer deletion on retroviral titre is represented by comparison of the density of 'northern dot blots' from viral RNA in supernatant derived from Δ-LTR vs WT-LTR constructs with the luciferase reporter gene. WT, vectors with unmodified LTR; Δ-LTR, vectors carrying enhancer deleted LTR. Basic = LTR driven vector without internal promoter driving the luciferase reporter gene in addition to WT-LTR or Δ-LTR are indicated. (Materials and Methods: 2.2.32).

As determined by the density of each dot blot pair (Δ-LTR vs WT-LTR construct) similar amounts of viral RNA genome were present in all the supernatants, which indicates that the enhancer deletion did not affect this.

There were however some differences in titer between the various promoter constructs, while a direct correlation between the titre estimated from eGFP constructs and the titre estimated by northern dot blot with a luciferase probe could not be drawn conclusively. These differences may simply account for differences in viral RNA processing of each promoter construct.

4.4.3 Retroviral Transduction with PINCO derived vectors

The Δ-LTR vector constructs harbouring the eGFP reporter gene were used to transduce NIH 3T3 cells (Figure 24). Flow cytometry for eGFP expression showed that, whilst titre on these murine fibroblasts was not severely affected, reporter gene expression from the Δ-LTR-CD11b construct was reduced by approximately two orders of magnitude (Figure 24, middle panels). The Δ-LTR-CMV construct showed a smaller reduction in expression that mainly revealed itself as a greater level of heterogeneity in eGFP fluorescence than seen when the LTR was also present in the vector (Figure 24, right panels).

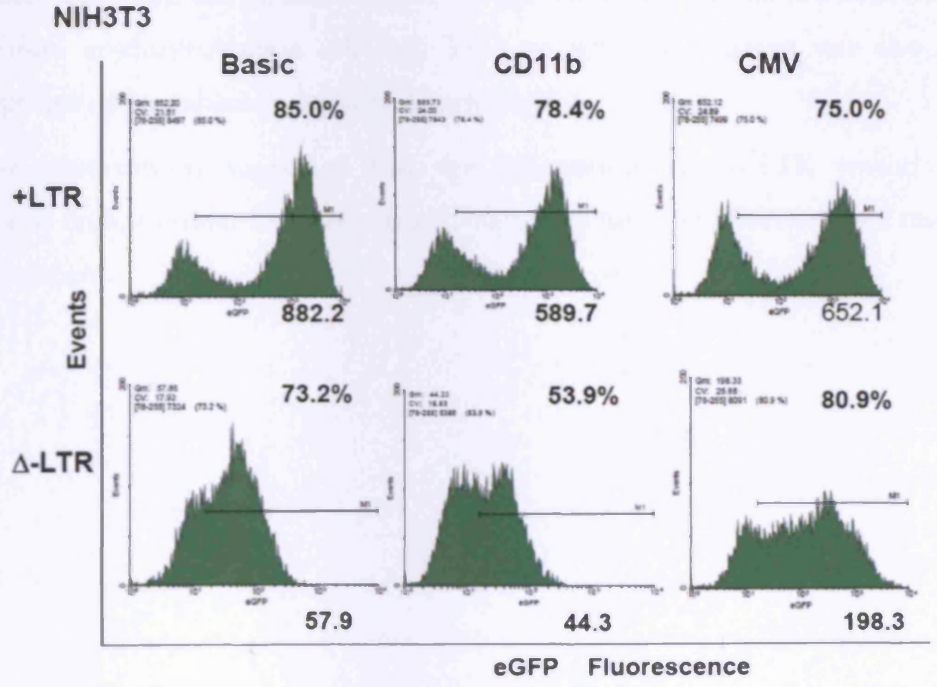


Figure 24: Effect of enhancer deletion on titre and transcription in NIH 3T3 cells.

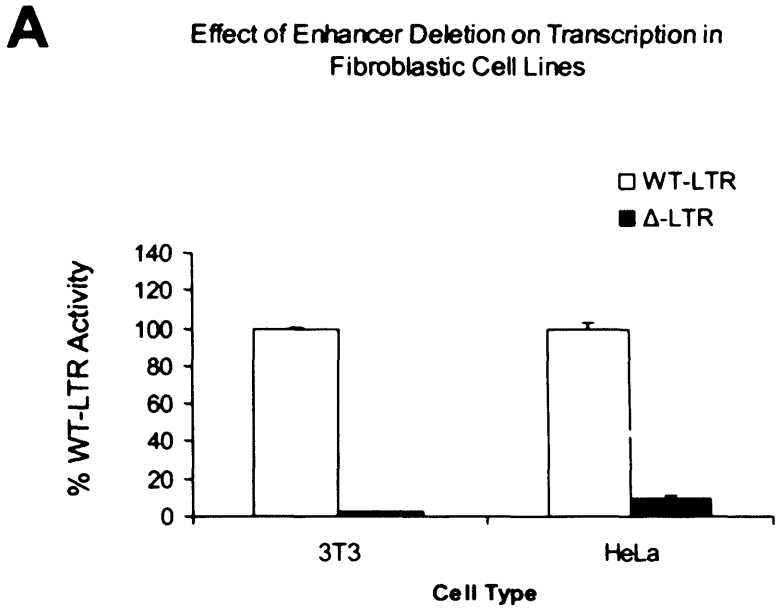
Retroviral transduction of NIH3T3 cells analysed by flow cytometry for EGFP expression. Fluorescent gates (M1) were set based on mock transduction with no virus (plot not shown, 1%). Wild type (+LTR), unmodified PINCO vector with intact LTR; Δ–LTR, enhancer-deleted vector. Basic = without internal promoter. CD11b = 732bp CD11b promoter. The percentage of positive cells is shown at the top right of each panel and mean fluorescent intensity (MFI) of the positive cell fraction is shown below the x-axis.

Chapter 4: Reporter Gene Expression in Cell lines transduced with retroviral vectors containing cellular promoters

To quantitate the effect of the enhancer deletion on transcription, equivalent 'wild-type' (PINCO-basic-luc, wt-LTR) and enhancer-deleted vectors (PINCOΔ-basic-luc, Δ-LTR) harbouring the luciferase gene in place of eGFP were used. Transcription in fibroblastic cells was again significantly reduced. Luciferase activity in both NIH3T3 and HeLa cells transfected with the Δ-LTR vectors demonstrated a significant reduction in comparison with the intact wt-LTR (Figure 25, A).

The major loss of reporter gene transcription could therefore be attributed to deletion of the retroviral enhancer. Interestingly however, when the 'basic' Δ-LTR vector (enhancer-deleted LTR and no internal promoter) was used to transduce myeloid cell lines, PLB 985 and U937, only a smaller loss of reporter gene activity, associated with enhancer deletion, was detected (Figure 25, B). However, this did not hold true for the immature erythroleukaemia cell line, TF-1, in which expression was also seriously compromised by enhancer deletion (Figure 25, B).

These observations suggested that the enhancer-deleted Δ-LTR vector, although seriously compromised for transcription, might still function effectively in a myeloid cell environment.



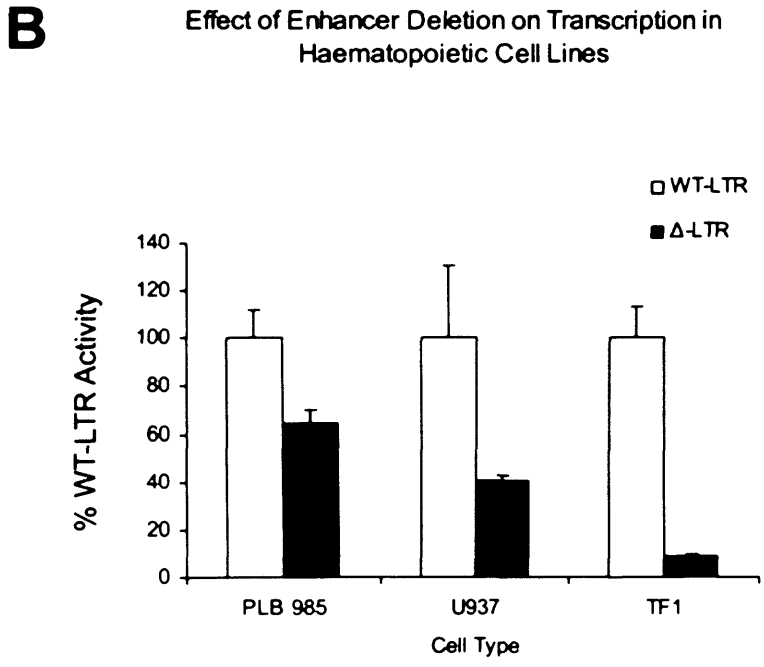


Figure 25: Tissue specificity and Effect of enhancer deletion on transcription in fibroblastic cell lines in comparison with transcription in haematopoietic cell lines.

Transcription from 'basic' retroviral vectors without exogenous promoter with unmodified PINCO basic luc WT LTR, white bars and enhancer deleted PINCOΔ basic luc Δ LTR, black bars in fibroblastic A and haematopoietic B cell lines. Data are mean ±SD from four independent transductions. All the differences between wild type and enhancer deleted vectors were significant at $p < 0.05$ Mann Whitney U test

4.4.4 Comparison of Cellular Promoters driving the Luciferase Reporter gene

In order to analyse and compare the performance of myeloid specific promoter constructs in myeloid cell lines, luciferase assays of transduced PLB965, U937 and TF1 cells (±PMA) were carried out.

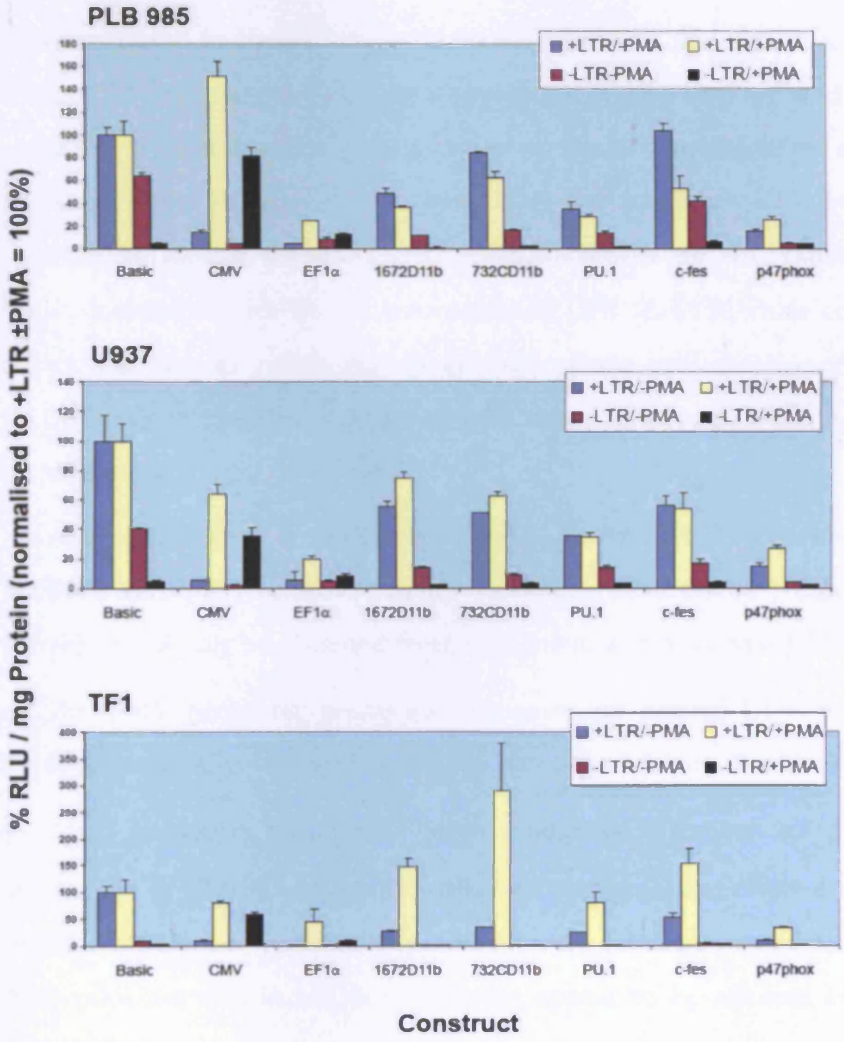


Figure 26: Luciferase activity by effect of PMA inducted cell differentiation on promoter constructs and enhancer deletion in transduced myeloid cell lines (PLB985, U937 and TF1).

Myeloid cells lines were transduced by spinocculation with retroviral vectors as indicated and induced ±PMA for 48h before assayed for luciferase activity as described in the Materials and Methods (2.2.24). Data are mean ±SD from four independent transductions and luciferase activity is presented as RLU/mg protein normalised to wild type LTR (+LTR) ±PMA = 100%. Normalisation is made in order to enable comparison between different cells line and under the

assumption that PMA has no effect on the transcription from the wild type LTR, that solely the effect of PMA on the internal promoter can be analysed. The blue bars represent wildtype LTR constructs without PMA (+LTR/-PMA), while the beige yellow bars represent the wild type LTR constructs with PMA (+LTR/+PMA). The purple bars represent enhancer-deleted LTR (ΔLTR/-PMA) constructs without PMA (ΔLTR/-PMA) and the black bars represent the enhancer-deleted LTR constructs with PMA (ΔLTR/+PMA).

In order to compare the luciferase activity data from induced and uninduced cells, the results in Figure 26 are presented by normalising all of the data to wild type LTR (±PMA) to 100%. This normalisation was based on the not unreasonable assumption that PMA has no effect on the transcription from the wild type LTR (+LTR) and permits not only to isolate the effect of PMA induction on the various internal promoter from constructs with the enhancer-deleted LTR (Δ-LTR) from confounding external factors, but also to enable the comparison of the performance of the same promoter in different transduced transduced cells lines, which may differ markedly in transduction efficiency.

While all myeloid promoter constructs with enhancer deleted LTR performed only poorly, in PLB985 and U937 cells, no activity was observed in the TF1 cell line, where luciferase activity could only be obtained from constructs with wild type LTR.

Interestingly, the CMV promoter construct with enhancer deleted LTR demonstrated potent inducibility in response to PMA in all cell lines tested (Figure 26, black column).

In contrast, PMA induction seemed to have a negative effect on all the myeloid promoter constructs in PLB985 and U937 cells, but no significant effect on the EF1α promoter, which as shown in previous experiments with transient assays, seemed to be inert to PMA induction and in addition does not appear to be affected by enhancer deletion of the LTR.

However, with regard to the other enhancer-deleted vectors with exogenous promoter, the evaluation seemed to be less conclusive with one exception presented by the CMV promoter showing a significant increase of luciferase activity in U937 and PLB985 cells when induced with PMA.

A number of other groups have also reported elevated levels of transcription from the CMV promoter in myeloid cell lines induced to macrophage differentiation. This effect however, could be indicative of the presence of a PMA responsive element in the CMV promoter sequence, rather than as a result of the induction of terminal cell differentiation (Chan et al., 1996; Sambucetti et al., 1989).

However, the time constraints and the costs of the materials precluded confirmation of these expression patterns.

4.4.5 Comparison of Cellular Promoters driving the eGFP Reporter gene

In parallel to the luciferase vectors, we also had created an equivalent set of promoter constructs with the eGFP reporter, which we wanted to evaluate by the use of various inducers of myeloid differentiation, including those directing differentiation towards the granulocytic lineage.

In particular, the human myelomonoblastic leukemia cell line PLB985 (Tucker et al., 1987) provides a good model to study myeloid differentiation. In fact, a $gp91^{phox}$ knockout cell line of PLB985 cells was the first to be developed to study X-CGD at the cell level (Zhen et al., 1993). The advantage of PLB985 cells is that they can be treated with retinoic acid (RA) or dimethylformamide (DMF) to induce granulocyte differentiation, or 12-myristate 13-acetate (PMA) to induce macrophage differentiation, respectively.

Hence we choose to evaluate our myeloid vectors with the eGFP reporter gene in the PLB985 cell first, although levels of transduction with U937 cells had shown to be more efficient. PLB985 cell lines were differentiated after transduction with eGFP vectors by treatment with 0.1μM phorbol 12-myristate 13-acetate (PMA) for 48h (macrophage differentiation) and by 60mM dimethylformamide (DMF) for an additional 24h (granulocyte differentiation).

Unfortunately, due to the time constraints of the project we had to precluded this part of our investigation too and with the availability of a set of $p47^{phox}$ deficient mice we prioritised preparations to test a new set of our vectors *in vivo*.

4.5 Discussion

In retrospect, we established that enhancer deletion of the LTR had minimal effects on titre (Table 3 and Figure 23), but a dramatic effect on the levels of transcription in fibroblasts (Figure 24 and Figure 25). Transduction efficiency into haematopoietic cell lines was significantly lower than fibroblastic cells, U937 cells being the most susceptible, and TF-1 being the most resistant and consequently, results from TF1 cells, in particular were inconclusive (Figure 26).

While enhancer deleted constructs performed poorly in transduced NIH3T3 and HeLa cells, possibly the most interesting finding was that our 'basic' enhancer deleted vector performed surprisingly well in haematopoietic cell lines (Figure 25). The mean fluorescent intensity (MFI) data (Figure 24) put the 'basic' vector at same level with CD11b, although the level of expression of the myeloid promoters in the myeloid cell lines was generally disappointing being much lower than expected and in addition showing little or no inducibility on myeloid differentiation (Figure 26).

Interestingly, the CMV promoter showed a peak response to PMA induction (Figure 26) as we were able to obtain already from the transient luciferase assays (Figure 10 and Figure 13) earlier, which confirmed observations made elsewhere (Chan et al., 1996; Sambucetti et al., 1989) that the CMV promoter might have a PMA responsive element in the CMV promoter sequence (Chan et al., 1996; Sambucetti et al., 1989).

Finally, it was concluded that the data obtained from transduced cell lines - after some time consuming and extensive analysis - were disappointingly inconclusive. None of the constructs harbouring myeloid promoters showed any real evidence of efficient myeloid cell specific transcription and none showed evidence for up-regulation in response to induced myeloid differentiation. It is not unreasonable to suggest that testing of promoter constructs under these conditions requires the selection of cell populations in which 100% of the cells are stably transduced. Without this criterion being satisfied, it is likely that there are too many variables to obtain reliably informative data, when the levels of expression are this low.

Stable transductants would allow the direct comparison of transcription following induction of differentiation over longer periods and multiple repeats, whilst avoiding the variability associated with multiple transductions.

Taking these data together, it was felt that the only reliable way to assay the potential of the myeloid promoter constructs would be to test them directly in animals. We were fortunate therefore that a knockout mouse model of $p47^{phox}$ recently developed at UCL became available to us (a kind gift of Dr. Jurgen Roes and Prof. Tony Segal) at the time (Jackson et al., 1995; Messina et al., 2002; Pollock et al., 1995; Segal et al., 1999), to enable further testing of our retroviral constructs.

5 Correction of p47phox knockout mouse model of CGD using enhancer-deleted vectors

5.1 Background: Murine models of CGD

To study different aspects of somatic gene therapy in CGD *in vivo*, several murine models have been developed for both X-linked ($gp91^{phox-/-}$) and an autosomal recessive ($p47^{phox-/-}$) form of CGD by gene targeting (Jackson et al., 1995; Messina et al., 2002; Pollock et al., 1995; Segal et al., 1999). These knockout mouse models show abnormalities in host defence and inflammation due to the lack of phagocyte superoxide production, and are vulnerable to infection with *Aspergillus* and *Staphylococcus*, representing a similar phenotype to that seen in human CGD, which confirms the importance of the respiratory burst for innate immunity (Goldblatt and Thrasher, 2000; Jackson et al., 1995; Pollock et al., 1995) . Therefore, these genetically engineered mice have become essential for *in vivo* studies of NADPH-oxidase function in phagocytic and non-phagocytic cells, the understanding of the role of superoxide in the host response to infection and inflammation and for the development of novel therapies.

In murine CGD, retroviral-mediated gene transfer into bone marrow cells has been shown to correct the respiratory burst oxidase activity in phagocytes *in vivo* and to improve the defect in the host defence against bacterial and fungal pathogens (Bjorgvinsdottir et al., 1997; Dinauer et al., 1999; Mardiney, III et al., 1997; Sadat et al., 2003; Schwickerath et al., 2004). These studies were some of the first to show that gene therapy could alleviate the clinical symptoms of an inherited disorder, using an animal model that closely resembles the human disease.

For our investigation a $p47^{phox-/-}$ deficient mouse was used as a disease model for CGD, which lacks phagocyte superoxide production, thus reflects an identical phenotype to that seen in human CGD. The $p47^{phox-/-}$ mouse (gene knockout, NCF-1) develops severe spontaneous bacterial and fungal infections similar to those developed by human CGD patients.

Chapter 5: Correction of p47phox knockout mouse model of CGD using enhancer-deleted vectors

5.2 Bicistronic retroviral Vector with therapeutic gene and eGFP marker

In order to restore superoxide generation in these mice our vectors were modified to incorporate the $p47^{phox}$ cDNA coupled by an internal ribosome entry sequence (IRES) of the encephalomyocarditis virus (ECMV) to the EGFP marker gene (see plasmid maps 8.9). These vectors make a single bicistronic mRNA transcript encoding both proteins, which are translated separately. The presence of the EGFP allowed for independent detection of transduced cells and the assessment of viral titre.

5.2.1 Cloning strategy of retroviral constructs for bicistronic expression

The cloning strategy and construction of the bicistronic retroviral vectors based on PINCO are described here briefly with the corresponding detailed restriction maps listed in the Appendix, paragraph 8.9,

In order to insert the p47cDNA from the vector pVZ1-NCF47k (kind gift from Dr Adam Rodaway, see plasmid map 8.6.1) into the bicistronic expression vector pIRES2-eGFP from Clontech (see plasmid map 8.8.1) the following subcloning steps were carried out: The p47cDNA was sub-cloned into the pCI-Vector (Promega, see plasmid map 8.7.1) using *Eco*RI and *Nhe*I sites, to create pCI-47cDNA (see plasmid map 8.7.5). Finally, p47cDNA-IRES2-eGFP (see plasmid map 8.8.3) was created by cutting the pIRES2-eGFP vector (Clontech, see plasmid map 8.8.1) with *Eco*RI and *Sal*I, whilst the p47cDNA fragment was taken *Eco*RI/*Sal*I from pCI-47cDNA (see plasmid map 8.7.5).

+LTR: (abbreviation for PINCO-Basic-p47cDNA-IRES2-eGFP, wild-type 3'LTR without CMV promoter, see plasmid map 8.9.1): To create +LTR-p47cDNA-IRES2-eGFP, the *Eco*RI/*Not*I fragment from p47cDNA-IRES2-eGFP (see plasmid map 8.8.3) was cloned into the *Eco*RI/*Not*I sites of PINCO (see plasmid map 8.5.1).

Δ-LTR (abbreviation for PINCO-Δ-Basic-p47cDNA-IRES2-eGFP, enhancer-deleted 3'LTR without CMV promoter, see plasmid map 8.9.2) was created by cloning the

*Eco*RI/*Not*I fragment from p47cDNA-IRES2-eGFP (see plasmid map 8.8.3) into PINCO-Δ-CMV-eGFP (equal to PINCO-Δ, see plasmid map 8.5.5).

Δ-LTR-CMV: (abbreviation for PINCO-Δ-CMV-p47cDNA-IRES2-eGFP, enhancer-deleted 3'LTR with CMV promoter, see plasmid map 8.9.3) was created by cloning the *Hind*III (blunted)/*Not*I cassette of p47cDNA-IRES2-eGFP (see plasmid map 8.8.3) into *Eco*RI (blunted)/*Not*I of PINCO-Δ-eGFP (see plasmid map 8.5.5).

To create *Δ-LTR-CD11b* (abbreviation for PINCO-Δ-732CD11b-p47cDNA-IRES2-eGFP, enhancer-deleted 3'LTR without CMV promoter plus 732bp of the CD11b promoter, see plasmid map 8.9.4), the *Eco*RI(blunted)/*Not*I fragment from p47cDNA-IRES2-eGFP (see plasmid map 8.8.3) was cloned into *Bam*I (blunted)/*Not*I digested Δ-LTR-732CD11b-eGFP(N1) (equal to PINCO-Δ-732CD11b-eGFP(N1), see plasmid map 8.5.32), which was created by sub-cloning the 732CD11b-eGFP *Bgl*II/*Not*I fragment from pN1-CMV-732CD11b-eGFP (see plasmid map 8.4.5) into the *Not*I/*Bam*HI viral vector backbone of Δ-LTR-eGFP (equal to PINCO-Δ-Basic-eGFP, see plasmid map 8.5.8). The latter was created by exchanging the CMV-eGFP cassette (*Not*I/*Bam*HI) of Δ-LTR-CMV-EGFP (equal to PINCO-Δ-CMV-eGFP, see plasmid map 8.5.5) with the EGFP (*Not*I/*Bgl*II) from pEGFP-N1 (Clontech). pN1-CMV-732CD11b-eGFP was created by truncating full length (1672bp) CD11b in pN1-CMV-1672CD11b-eGFP with a *Sac*I/*Sac*I digest and religation. pN1-CMV-1672CD11b-eGFP (see plasmid map 8.4.3) was sub-cloned by inserting the *Hind*III/*Bam*HI CD11b promoter from pΦGHCD11b (see plasmid map 8.1.7, kind gift from Dr. D. Hickstein) into the *Bam*HI/*Hind*III side of pEGFP-N1 (Clontech).

Δ-LTR-EF1α (abbreviation for PINCO-Δ-EF1α-p47cDNA-IRES2-eGFP, enhancer-deleted 3'LTR without CMV promoter, but the EF1α promoter instead, see plasmid map 8.9.7) was created from PINCO-Δ-EF1α-eGFP (see plasmid map 8.5.20) by *Eco*RI/*Not*I digest and insertion of the bicistronic unit from p47cDNA-IRES2-eGFP (see plasmid map 8.8.3) via the same restriction enzymes.

Finally, Δ-LTR-p47 (abbreviation for PINCO-Δ-p47phox-p47cDNA-IRES2-eGFP, enhancer-deleted 3'LTR without CMV promoter, but the p47phox promoter instead,

see plasmid map 8.9.5) was achieved by using the vector PINCO-Δ-p47phox-eGFP (see plasmid map 8.5.36) as backbone with a *Eco*RI/*Not*I digest and insertion of the the bicistronic unit from p47cDNA-IRES2-eGFP (see plasmid map 8.8.3) via the same restriction enzymes.

The same cloning strategy was used for +LTR-p47 (abbreviation for PINCO-p47phox-p47cDNA-IRES2-eGFP, wild-type 3'LTR without CMV promoter, but the p47phox promoter instead, see plasmid map 8.9.6) with the PINCO-p47phox-eGFP plasmid (see plasmid map 8.5.35) as backbone.

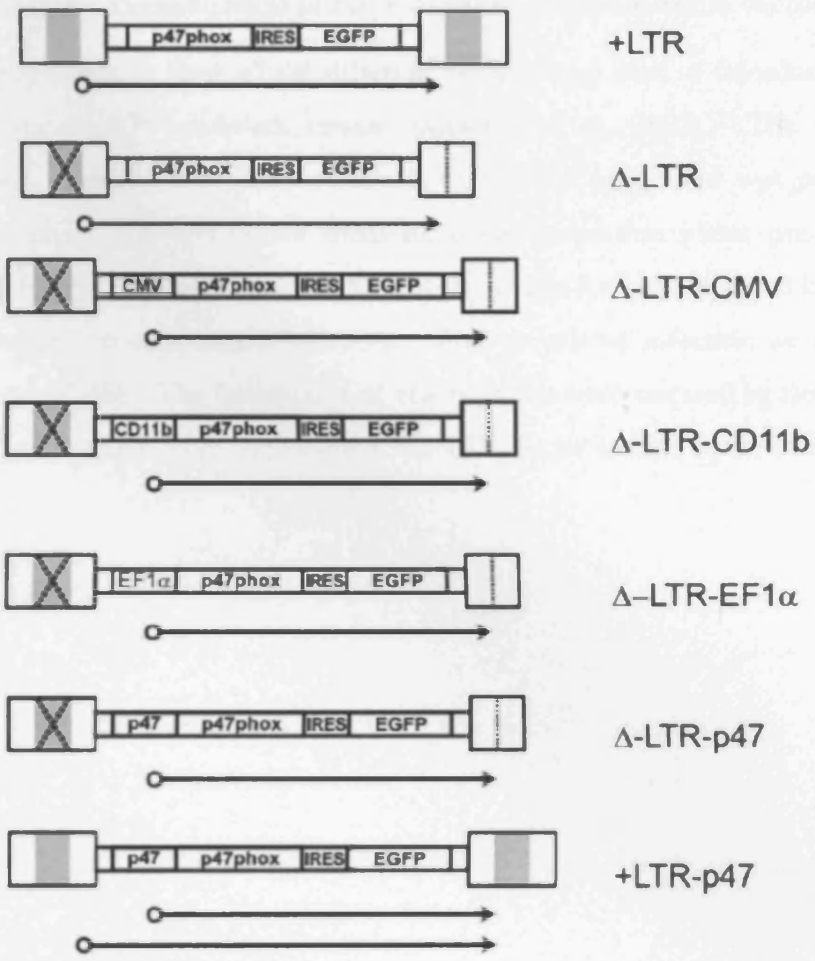


Figure 27: Schematic diagrams of PINCO derived bicistronic retroviral vectors for the transduction and reconstitution of $p47^{phox}$ knockout mice.

The expanded box at each end represents the LTR in which the grey area identifies the 75-bp enhancer repeat region. The enhancer deletion in the 3`LTR is indicated by the dotted line and the cross in the 5`LTR indicates that the deleted LTR will be copied and transposed to the 5`end in the provirus. The arrows underneath the vector schematics indicates the $p47^{phox}$cDNA-IRES2-eGFP transcription unit. Detailed plasmid maps are listed in the Appendix 8.9.

Above bicistronic vectors were used to transduce bone marrow of $p47^{phox-/-}$ donor "knockout" mice and analysed for transfection efficiency.

5.2.2 Retroviral Transduction of bone marrow with bicistronic vectors

Retroviral supernatants from all the different vectors were used to transduce total bone marrow from a $p47^{phox}$-deficient mouse (Messina et al., 2002). The transduction protocol was derived from one developed for clinical usage and was performed in cytokine-supplemented serum-free medium, using micro-titre plates pre-coated with fibronectin fragment (Schilz *et al.*, 1998). Infection was further enhanced by the use of "spinocculation" to concentrate retrovirus. Five rounds of infection were performed over a period of 48h. The initial rates of transduction were assessed by flow cytometry (Figure 28) and ranged from 57% for the WT-LTR vector to 19% for the CD11b vector.

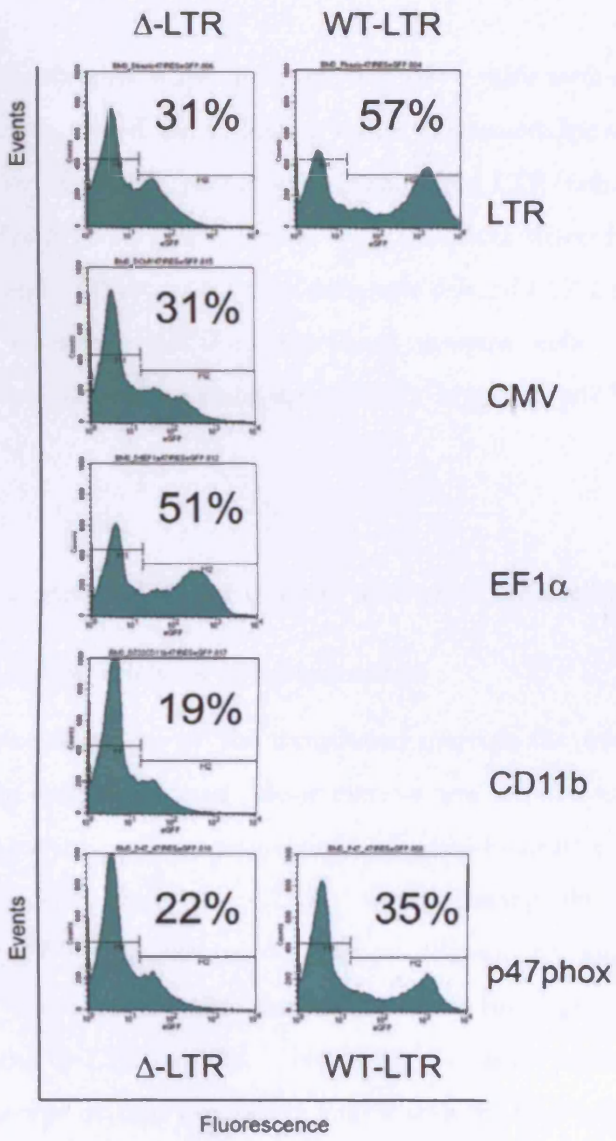


Figure 28: Flow cytometric analysis of transduced bone marrow cells from 47$^{-/-}$ 'knockout' donor mice following transduction (before transplant).

Transduction efficiency of bicistronic vectors are indicated by FACS histograms as described in the Materials and Methods (2.2.35.1 and 2.2.35.2) and abbreviated as follows: Δ-LTR, vector with enhancer deleted LTR (left panel); WT-LTR, vector with unmodified LTR/enhancer (right panel) both without internal promoter; CMV, vector with enhancer deleted LTR and CMV as internal promoter; EF1α, vector with enhancer deleted LTR and EF1α as internal promoter; CD11b, vector with enhancer deleted LTR and CD11b as internal promoter; p47^{phox}, vector with enhancer deleted LTR (left panel) and unmodified WT-LTR (right panel) both with p47phox as internal promoter. The marker was placed using untransduced cells such that 99% were left of the marker. The percentage of EGFP-positive cells is shown at the top right of each panel.

Chapter 5: Correction of p47phox knockout mouse model of CGD using enhancer-deleted vectors

Due to the limited availability at the time, only ten p47$^{-/-}$ mice were available for analysis, it was decided to make use of the following vector constructs for transplantation in the p47^{phox}-defcient mice: WT-LTR, vector with unmodified LTR/enhancer; Δ-LTR vector with enhancer deleted LTR; CMV, vector with enhancer deleted LTR and CMV as internal promoter and CD11b, vector with enhancer deleted LTR and CD11b as internal promoter. The remainder of the transduced marrow cells from these selected constructs was therefore used to transplant lethally irradiated p47^{phox}-deficient recipient mice.

5.3 Detection of retroviral vector in bone marrow of reconstituted mice

5.3.1 Flow cytometric analysis of bone marrow

Six weeks after transplantation of the transduced marrow, the mice were analysed for the presence of the retroviral vector. Bone marrow was isolated and analysed by FACS for eGFP fluorescence. The proportion of eGFP-positive cells ranged from approximately 4.3-13.0% with the CD11b vector having the lowest transduction frequency and the WT-LTR vector the highest (Figure 29, left-hand panels). Not unexpectedly, the intact LTR vector showed evidence for a generally higher level of expression than the Δ-LTR vectors. However, we were particularly interested to compare the phenotype of cells expressing EGFP with the total marrow population.

Bone marrow cells were isolated from the transduced recipients and subjected to analysis by flow cytometry using antibodies identifying myeloid cells, these were: Gr-1, which stains mainly granulocytes and mature macrophages; F4/80, a macrophage marker; and CD11b itself, which is present on most phagocytes, particularly neutrophils. For this analysis, the total population of stained cells (Figure 29, right-hand panels, 'total BM') was compared with the antibody staining of cells that were gated for EGFP expression and hence contained the vector genome (Figure 29, middle panels, marked 'EGFP+').

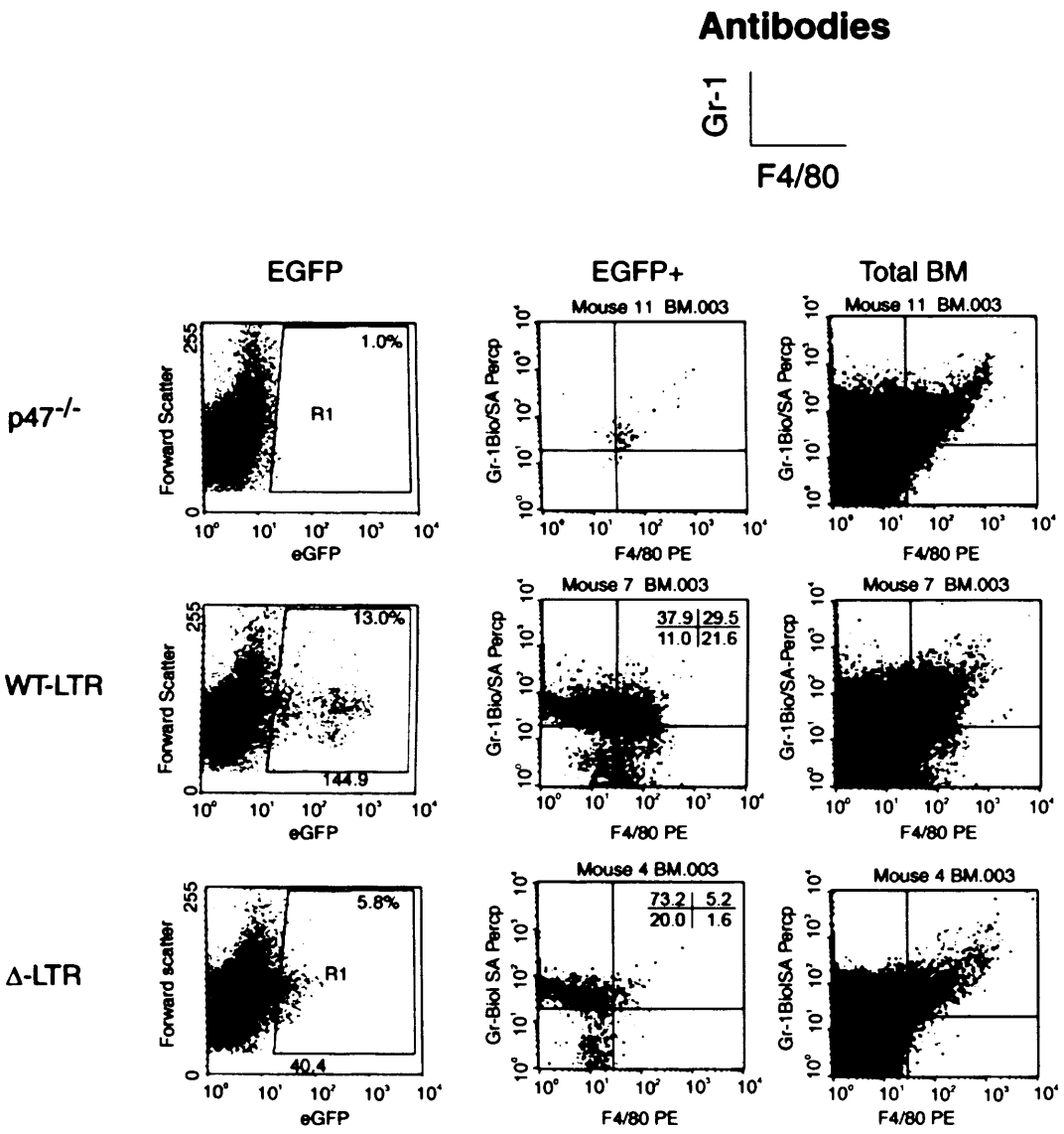


Figure 29: Flow cytometric analysis of transduced bone marrow cells from reconstituted p47$^{-/-}$ mice.

Bone marrow (BM) cells from mice transplanted with retrovirally transduced cells were analysed for EGFP fluorescence and by myeloid-cell-specific monoclonal antibodies as described in the Materials and Methods (2.2.35). Left-hand panels show total EGFP fluorescence in the bone marrow of transplanted mice and the percentage of positive cells and MFI is indicated. Panels marked EGFP+ show antibody staining of cells gated for positive EGFP fluorescenc and GR-1 vs F4/80 of EGFP+ cells (middle panels).. Total BM (right-hand panels) show antibody-staining pattern of whole, ungated population. p47$^{-/-}$, shows marrow from recipient p47^{phox}-deficient mouse; WT-LTR and Δ-LTR, shows marrow cells from mice transduced using wild-type and enhancer-deleted vectors as indicated previously. Summary of quantitative data from FACS dot blots of all constructs are represented in Table 4.

The data for all the constructs are summarised in Table 4.

	Gr-1+		F4/80+		Gr-1+/F4/80+		-/-		CD11b+		
	EGFP	Total	EGFP	Total	EGFP	Total	EGFP	Total	EGFP	Total	*p=
$P47^{+/+}$	-	51.9	-	14.2	-	11.9	-	45.8	-	68.5	
$p47^{-/-}$	-	58.4	-	7.6	-	6.7	-	40.7	-	64.5	
+LTR (1)	**67.4**	63.2	**51.1**	11.3	**29.5**	8.8	11.0	34.3	**76.4**	71.8	*0.025*
+LTR (2)	**71.7**	67.3	**67.5**	22.1	**45.4**	16.3	6.1	26.9	**84.3**	75.8	
Δ-LTR (1)	**78.4**	72.8	**6.7**	5.6	5.2	5.3	20.0	27.9	**83.7**	80.2	*0.025*
Δ-LTR (2)	**66.3**	58.5	**10.6**	6.6	**6.7**	5.8	29.9	40.7	**72.9**	60.8	
CD11B (1)	**81. 5**	73.5	**11.2**	4.6	**8.8**	4.1	16.1	26.0	**85.4**	80.6	*0.025*
CD11B (2)	**67.3**	61.3	**11.9**	6.7	**9.8**	6.1	30.6	38.0	**75.6**	71.6	
CMV (1)	**69.4**	66.0	**6.5**	3.0	**4.6**	2.7	22.9	33.8	**75.2**	66.0	*0.025*
CMV (2)	**64.2**	60.0	**9.7**	9.1	6.5	8.4	32.5	39.3	**75.3**	67.2	
**p=	*0.005*		*0.005*		*0.05*		*0.005*		*0.005*		

Table 4: Distribution of myeloid markers in EGFP positive and total marrow cells.

Values shown are percentage of cells staining positive for the myeloid cell markers. -/- are cells that did not stain with either Gr-1 or F4/80. Values are shown for cells gated for EGFP expression and for the total bone marrow. Where the value in the EGFP+ population is a higher proportion than the proportion in the total marrow, the figures are shown in bold. Where the value in the EGFP+ population is a lower proportion than the proportion in the total marrow the figures are in grey and bold. *p values were calculated for each construct using the three different myeloid markers only, using the Wilcoxon signed-rank test. **P values were calculated for each marker, or combination, using the Wilcoxon signed-rank test.

Somewhat unexpectedly, with all the vectors, including the WT-LTR vector, we detected a modest preference for myeloid cell expression; compared with total marrow, a slightly greater proportion of the EGFP-positive population was also positive for the three myeloid-specific, surface markers (Gr-1 and F4/80; Figure 29, top left, top right and bottom right quadrants; see also Table 4). There was also a concomitant reduction in the number of cells that were negative for both Gr-1 and F4/80 (Figure 29, bottom left quadrants). This pattern was most striking for the WT-LTR vector, which, unlike the other constructs, was also associated with a significant increase in the population of

Gr-1+/F4/80+ cells (Figure 29, top right quadrant and Table 4). These were most probably mature macrophages, although why they were not represented with the enhancer-deleted vectors is unclear at present.

5.3.2 Colony assays

Some of the remaining marrow was used for plating in semi-solid medium. Haematopoietic colonies were assayed after 14 days for the presence of the retroviral vector by analysis under a fluorescence microscope. Retrovirally transduced colonies, absent from the untransplanted mice (Figure 30), were clearly detected by bright green fluorescence, indicating the presence of EGFP in the cells.

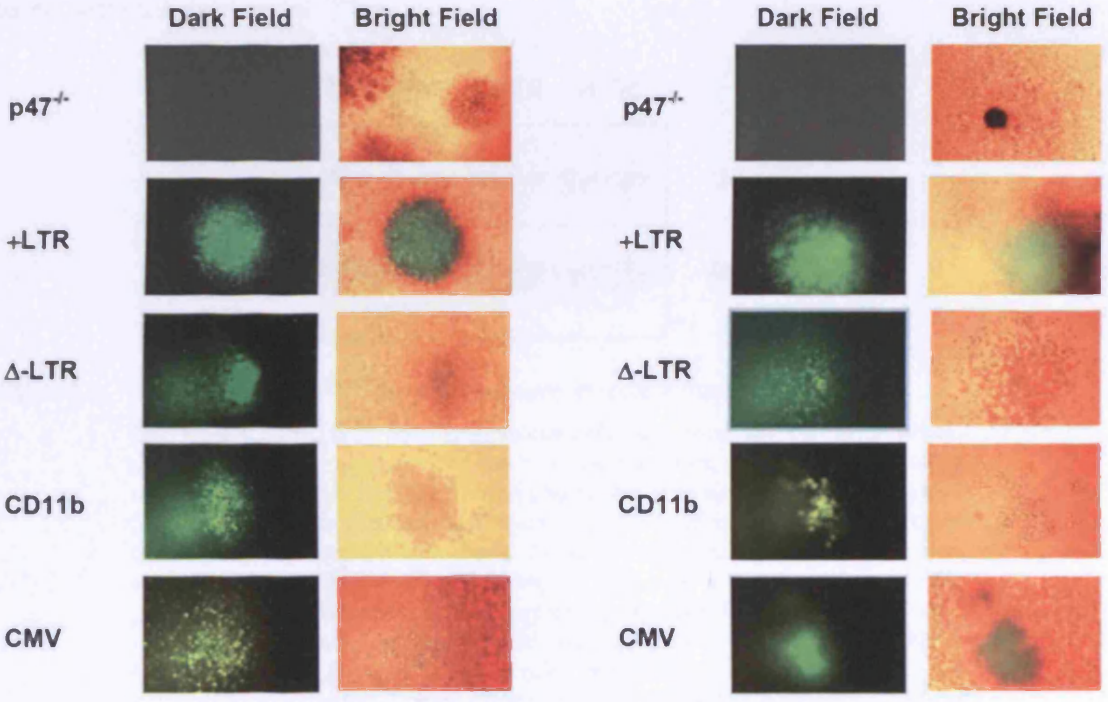


Figure 30: Colony assays: EGFP expression in reconstituted p47$^{-/-}$ mice.

Bone marrow cells were plated in semi-solid culture medium in the presence of cytokines and colonies were allowed to develop for a further 14 days as described in the Materials and Methods (2.2.36). The colonies were analysed for the presence and expression of the retroviral genome by observation of EGFP fluorescence under an inverted UV microscope. Genotype and retroviruses as identified previously.

The intact LTR vector produced the brightest colonies (Figure 30), though a wide variation was observed with all the constructs. Despite a generally reduced level of fluorescence, fluorescent colonies were also readily detected with the Δ-LTR vector (Figure 30).

5.4 Analysis of p47*phox* expression in tissues of the reconstituted mice

5.4.1 Western blot analysis of spleen and bone marrow

To begin the analysis of $p47^{phox}$ expression, the spleens were removed from the transplanted mice and assayed for presence of $p47^{phox}$ protein by Western blot. Proteins separated by 10% SDS-PAGE were transferred to a nitrocellulose membrane and incubated with anti-human $p47^{phox}$.

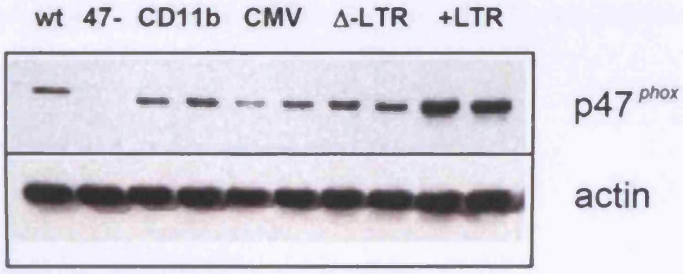


Figure 31: Western Blot of $p47^{phox}$ protein expression in reconstituted $p47^{-/-}$ mice.

The presence of $p47^{phox}$ protein in spleen cells (and bone marrow, same results not shown due to bad quality of the blot resulting from not enough material as marrow were needed for other experiments) was demonstrated by Western blot (as described in the Materials and Methods, 2.2.39) of total cell protein probed with anti-$p47^{phox}$ peptide antiserum. Equal protein loading in each lane was confirmed by stripping and re-probing the blot with antibody to β-actin. Wt, proteins from wild-type mouse, expressing normal levels of $p47^{phox}$; 47-, recipient $p47^{-/-}$ knockout mouse with no endogenous expression of $p47^{phox}$; CD11b, proteins from two $p47^{-/-}$ mice transplanted with transduced bone marrow using enhancer-deleted vector driven from CD11b promoter; CMV, as before but with CMV-driven enhancer-deleted vector; Δ-LTR, Δ-LTR-driven vector; +LTR, unmodified LTR-driven vector.

No protein was detectable in the untransplanted recipients (Figure 31, lane 47-). In all cases, however, $p47^{phox}$ could be detected in the transplanted mice. The Δ-LTR vector (Figure 31, lane Δ-LTR) proved to have levels of $p47^{phox}$ protein significantly greater

than either the CD11b- or CMV-driven vectors (Figure 31, lanes CD11b and CMV, respectively), although the animals transduced with an intact LTR (Figure 31, lane +LTR) had the highest levels of $p47^{phox}$ expression.

5.4.2 NBT analysis of bone marrow derived colonies

Having established that myeloid cells in the mouse bone marrow were efficiently transduced by the enhancer-deleted vectors, we investigated whether $p47^{phox}$ function had been restored to these cells by assaying their ability to generate superoxide. Bone marrow cells from the transplanted mice were plated into semi-solid medium and myeloid colonies arising at day 14 of culture were assayed for the generation of superoxide by the addition of nitroblue tetrazolium (NBT). The reduced form of this compound forms a dark-blue insoluble precipitate which can be used to detect the presence of a functional NADPH oxidase in myeloid colonies (Sekhsaria *et al.*, 1996).

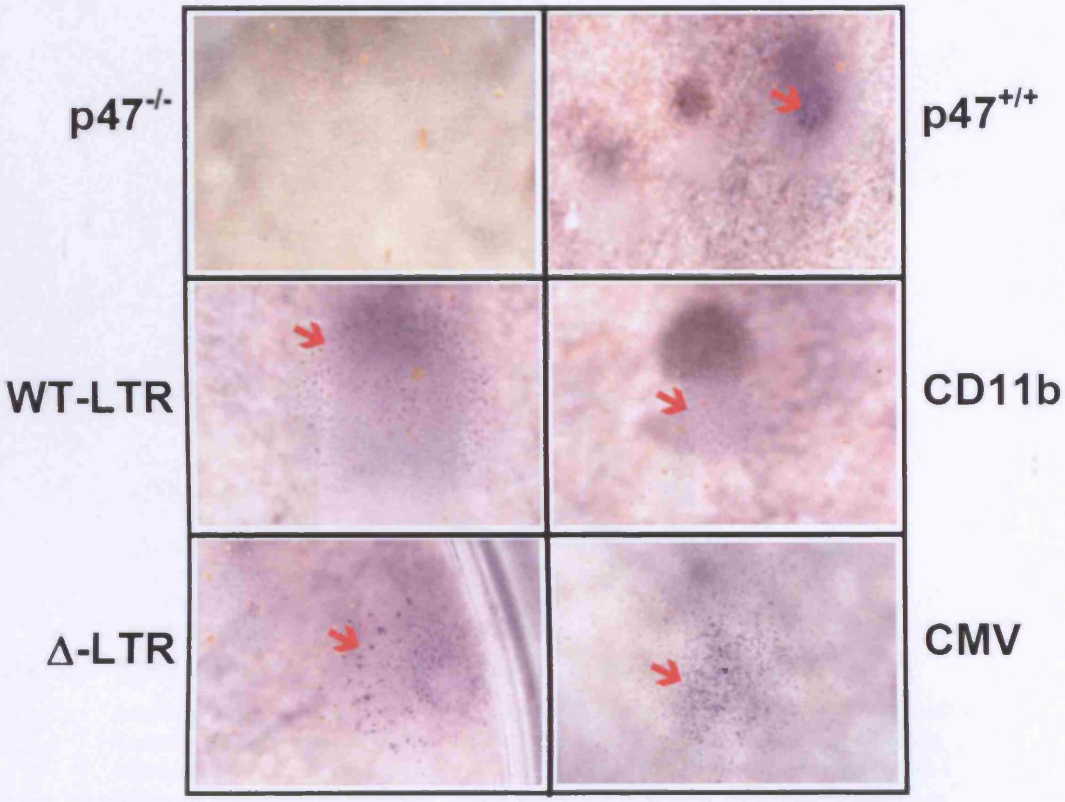


Figure 32: Colony Assays: NBT Analysis of haematopoietic colonies for NADPH oxidase activity in reconstituted $p47^{-/-}$ mice.

Oxidase activity was detected by *in situ* staining of bone marrow derived haematopoietic colonies using NBT as described in the Materials and Methods (2.2.36 and 2.2.37). Oxidase activity is revealed by the presence of a dark-blue precipitate in the cells. Examples of positive colonies are shown by red arrows.

It is noteworthy that with all the vectors blue-staining colonies were detected (Figure 32), that were clearly absent in the untransduced recipients (Figure 32, top right panel).

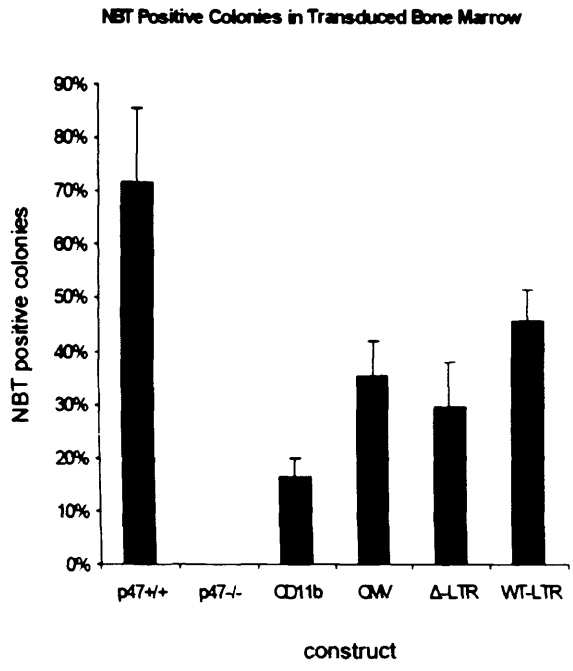


Figure 33: Percentage of colonies in transplated mice scoring positive with NBT.

Oxidase activity was detected by *in situ* staining of bone marrow derived haematopoietic colonies using NBT as shown in Figure 32 and described in the Materials and Methods (2.2.36 and 2.2.37). NBT positive colonies were scored for each construct as indicted.. Data are mean ±SD for two mice analysed in duplicate.

Enumeration of positive colonies (Figure 33) again showed that the highest proportion were found in the mice transduced with the WT-LTR vector, where nearly 50% of the colonies scored positive compared with approximately 70% positive colonies in normal p47$^{-/-}$ mice. Significant numbers of colonies showing evidence for superoxide generation were found in cells from all the mice transduced with the Δ-LTR vector still giving rise to over 30% positive colonies.

5.4.3 Superoxide generation in peripheral blood

To complete this analysis we also investigated superoxide generation in the peripheral blood of these animals. For this we employed an assay for superoxide depending on the reduction of dihydrorhodamine (Emmendorffer et al., 1990; Emmendorffer et al., 1994) which is detected as a shift in the red fluorescence of rhodamine and can be analysed by

FACS. To calibrate the assay a standard curve was generated using mixtures of white blood cells from normal and $p47^{phox}$-deficient mice (Figure 34).

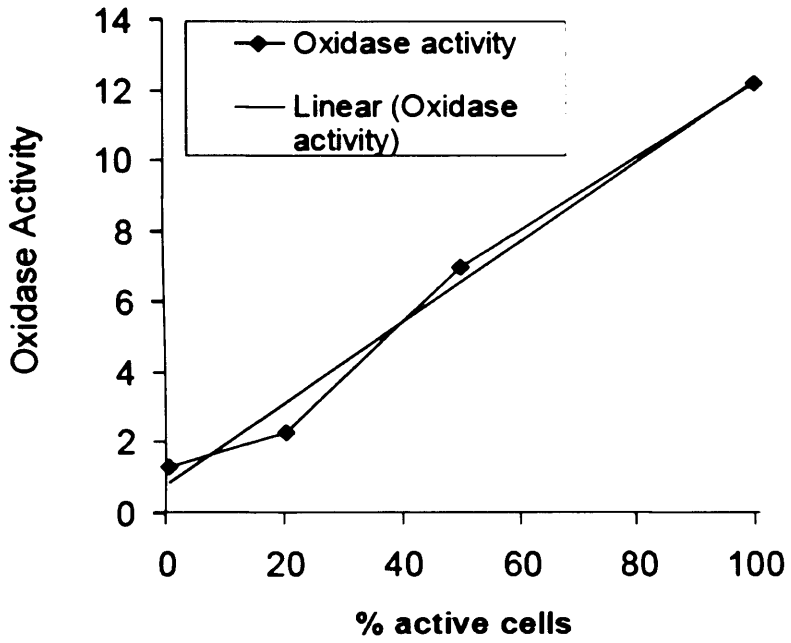


Figure 34: Calibration of NADH oxidase activity.

A standard curve was produced by mixing blood in varying ratios from wild-type and $p47^{-/-}$-deficient mice and the resulting oxidase activity was plotted against the percentage of wild-type cells as described in the Materials and Methods (2.2.38). The regression analysis was used to calculate the values shown in Figure 35

Somewhat surprisingly, very high levels of superoxide generation were detected in the transduced cells, though we currently have no explanation for the disparity in oxidase activity between the mice reconstituted with the CD11b. We suspect, however, that the lower value is more realistic as the higher estimate was from a sample compromised by a poor recovery of cells. In the case of mice transduced with the WT-LTR vector, supra-normal levels of superoxide generation were detected (Figure 35). More unexpectedly, perhaps, was the fact that superoxide generation levels comparable to those of normal cells were also obtained from Δ-LTR vector transduced cells, which gave the best results of all the enhancer-deleted vectors (Figure 35).

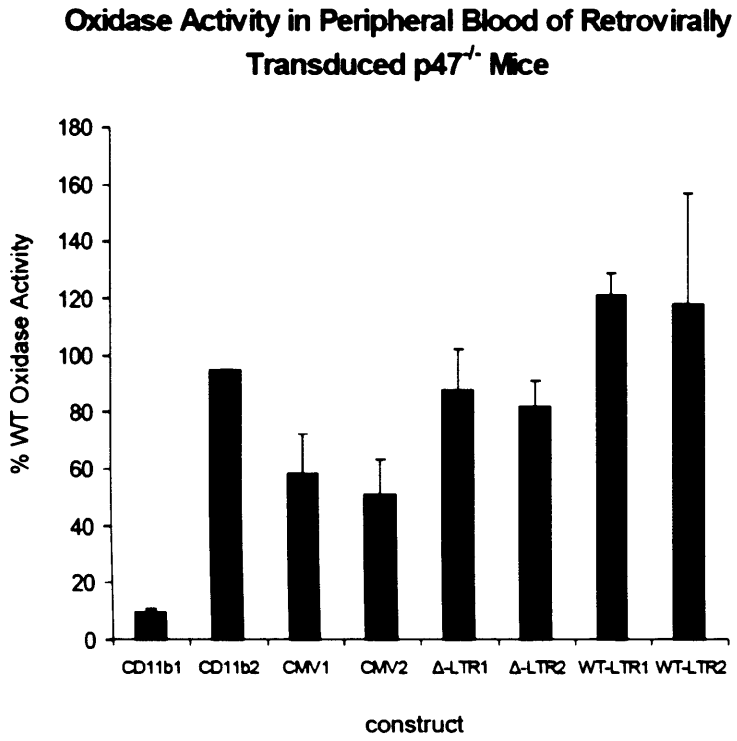


Figure 35: DHR assay of NADPH oxidase activity in eGFP-gated peripheral blood cells of reconstituted mice.

Dihydrorhodamine (DHR) assay was carried out as described in the Materials and Methods 2.2.38. Values are mean ±SD (n=4) and are shown specifically for eGFP+ peripheral blood cells of each reconstituted mouse, identified by the retroviral vector used.

This last observation is of particular relevance to the treatment of CGD, as it is well established that relatively low numbers of cells able to generate normal levels of superoxide can provide protection from infection (Roos, 1994; Segal et al., 2000), whereas cells with significant levels of partial activity, even when present in normal numbers (Bu-Ghanim et al., 1995), are unable to effectively kill engulfed pathogens. The high levels of superoxide generation we observed in mice transduced with our Δ-LTR vector, coupled with its freedom from viral enhancer elements, make it a highly promising candidate for creating a new generation of gene therapy vectors with a materially improved safety profile.

5.5 Discussion

We have described here the construction of an enhancer-deleted vector suitable for application in gene therapy protocols for treating diseases that affect the myeloid lineage. Although the theory and practice surrounding enhancer-deleted and self-inactivating vectors has been known for some time (Yu et al., 1986), only recently, especially with the increased levels of interest in HIV-derived lentiviral vectors, have their attributes been considered to be of primary importance (Kraunus et al., 2004; Schambach et al., 2006; Thornhill et al., 2008).

The most commonly encountered problem with the use of enhancer-deleted or self-inactivating (SIN) vectors has been loss of titre (Miller and Whelan, 1997; Soriano et al., 1991; Yee et al., 1987; Yu et al., 1986). Interestingly, the constructs we have described here do not appear to suffer from this problem (see chapter 4, 4.4.2), but the reasons for this are not abundantly clear. It may be that simple deletion of the 3`LTR enhancer 75-bp repeats, leaving the promoter essentially intact, helps to maintain efficient polyadenylation of retroviral genomic RNA. Moreover, the absence of a second promoter or enhancer, active in the packaging cells in addition to the LTR, might help to maintain titre by avoidance of interference or competition between the internal promoter and the LTR. Such competition between promoters could also partially account for the failure of the CD11b promoter to enhance transcription over Δ-LTR in myeloid target cells.

Alternatively, it may be that the loss of titre attributed to other SIN vector constructs can actually be accounted for by reduced levels of transcription in the target cells used for titration. By estimating titre using dot blots we have shown that some apparent loss of titre in the enhancer-deleted vectors is really due to poor levels of transcription in 3T3 cells when expression is driven from a promoter more active in myeloid cells. Where tissue-specific promoter elements are employed, it may be generally safer to estimate titre using a method that is independent of retroviral-mediated gene expression like dot blotting, or, better still, real-time PCR.

Chapter 5: Correction of p47phox knockout mouse model of CGD using enhancer-deleted vectors

Perhaps the major conundrum in the work we have described has been the high level of oxidase activity restored by the Δ-LTR vector, which has no enhancer and only a retroviral promoter. The precise boundaries of the enhancer deletion in our constructs have led us to focus on a transcription factor binding site homology still present in the enhancer-deleted LTR; this is a site for the transcription factor family C/EBP (see Figure 36, red arrow), which lies just downstream of the *Xba*I site (Figure 36).

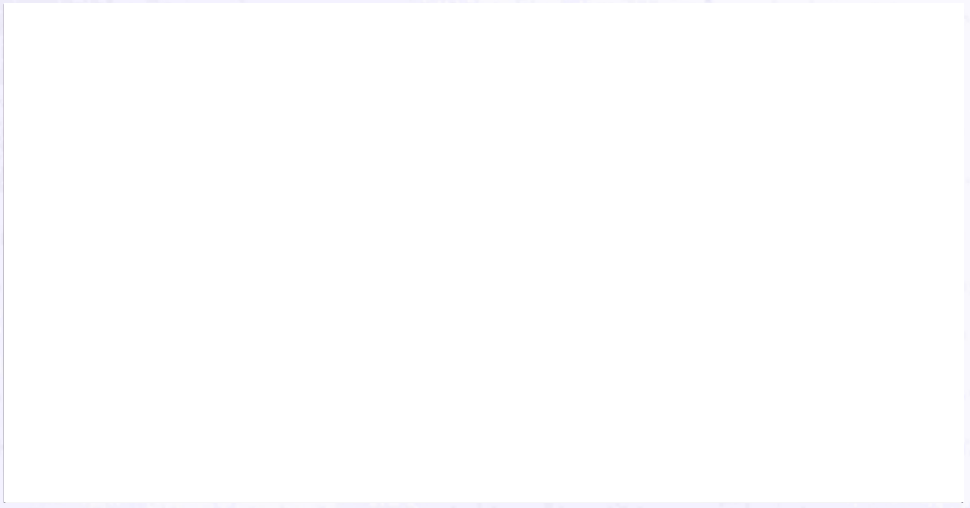


Figure 36: Detailed schematic representation of retroviral LTR.

Detail from the retroviral LTR showing the enhancer deletion between *Nhe*I and *Xba*I, which removes both of the 75-bp repeat sequences containing a number of transcription factor binding sites as shown and described previously in Figure 20. The deletion leaves the TATA element of the retroviral promoter and transcriptional start point intact, but also a C/EBP binding site as indicated by the red arrow (adapted from (Coffin et al., 1997)

The critical role of C/EBP binding factors in myeloid cell differentiation, particularly C/EBPα, has been well documented (Friedman et al., 2003; Kummalue and Friedman, 2003; Nagamura-Inoue et al., 2001). In fact the differential expression of C/EBPα appears to influence the lineage choice of bi-potent G/M precursors to either granulocytic or monocytic direction (Zhu and Emerson, 2002), while C/EBPα was found preferentially expressed in and required for the granulocytic lineage, but not for the monocytic lineage (Zhang et al., 1997).

6 Discussion and Conclusions

6.1 General Discussion

Improvements in retroviral vectors, haematopoietic stem cell transplantation techniques and transduction protocols have lead to the achievement of successful gene therapy protocols for the inherited immunodeficiency diseases X-linked severe combined immune deficiencies (SCID-X1)(Cavazzana-Calvo et al., 2000a; Thrasher et al., 2006) and adenosine deaminase deficient SCID (ADA-SCID)(Aiuti et al., 2002; Gaspar et al., 2006) using retroviral vectors. More recently some therapeutic benefit has also been achieved for patients suffering from X-linked chronic granulomatous disease (X-CGD)(Ott et al., 2006), with the promise that gene therapy may become a standard treatment for this disease in the future. However, a number of factors limiting the effectiveness and safety of this treatment are still under investigation and will require further development before gene therapy for CGD becomes the treatment of choice.

One important consideration where CGD differs from SCID is that alternative and established methods of treatment for CGD patients are available: the use of prophylactic antibiotics and interferon-γ (IFN-γ), coupled with treatment of acute infections and prolonged courses of antimicrobial treatment have significantly improved the clinical course of patients with CGD (Dinauer, 2005). With life expectancies of at least 25 plus years for the majority of CGD patients, for gene therapy to be an acceptable treatment in CGD it is essential that it is deemed both safe and efficient before it can be used in routine clinical application.

Concerns over the safety and limitations of the current of technologies retroviral-mediated gene therapy have been brought sharply into relief in recent years, from the experience of Fischer and his colleagues following an otherwise highly successful clinical trial (Cavazzana-Calvo et al., 2000b; Hacein-Bey-Abina et al., 2002b), of gene therapy for X-linked severe combined immune deficiency (X-SCID) (Hacein-Bey-Abina et al., 2003b; Hacein-Bey-Abina et al., 2003c; Kaiser, 2003; Kohn et al., 2003). A number of patients in this trial developed a gene therapy-induced leukaemia owing to

the random nature of retroviral integration. In every case, this leukaemia arose from integration of the retroviral genome in close proximity to the LM02 gene, a gene involved in the control of T-cell proliferation and known to have the capacity to be oncogenic.

The potential risks from random retroviral integration have been well documented (Cornetta et al., 1991) and previously have been accepted as finite but fairly remote. However, the experience gained in the X-SCID clinical trial has renewed the safety concerns over the use of retroviral vectors in clinical gene therapy protocols.

As revealed by the X-SCID incident, the chief risk arising from random integration is leukaemogenesis caused by insertional mutagenesis leading to inactivation of a tumour suppressor gene, or – as was the case in the X-SCID trial of Fischer and co-workers (Hacein-Bey-Abina et al., 2003b; Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003c) – the activation of an oncogene.

Furthermore, the recent successful correction of X-linked CGD was revealed to be absolutely dependent on the expansion of multiple haematopoietic clones carrying insertions that either occurred in or near PR domain-containing zinc-finger gene loci. Their predominance in the myeloid blood compartment also suggested that clonal expansion originated from an immature myeloid progenitor rather than a stem cell (Ott et al., 2006; Ott et al., 2007). Once again, some of the genes involved were of known oncogenic potential and this indicates that extreme caution is required in the further use of this gene therapy approach even in a potentially life-threatening disease such as CGD.

Both experiences with these recent advances in the use of gene therapy have therefore necessitated a reassessment of such the risks. Hence, novel vector design, safety issues and possible solutions are today high on the agenda in the field of gene therapy and have been the subject of a number of reviews (Baum et al., 2003; Baum et al., 2006; Haviernik and Bunting, 2004; Sinn et al., 2005; Yi et al., 2005).

One proposed strategy to reduce the risks inherent in the use of gene therapy involves co-transduction of a "suicide–gene" under the control of an inducible promoter, as an important safety feature, since destruction of transduced cells can potentially be

triggered if abnormal growth is observed (Painter et al., 2005). In addition, the conditional expression of the transgene restricted to the appropriate target cells via the combination of targeted transduction, cell type-specific expression, and targeted local administration could help to increase the overall safety of retroviral vector systems.

Further, splitting of the viral genome, use of self-inactivating (SIN) retroviral vectors, or complete removal of the coding sequences for *gag*, *pol*, and *env* genes are all desirable strategies to employ in the production of retroviruses for clinical usage, to virtually eliminate the possibility of generating replication competent retroviruses (RCR), which could contaminate clinical retroviral preparations.

Lately, the use of novel non-integrating lentiviral vectors has been proposed as a solution to overcome insertional effects of the virus (Philpott and Thrasher, 2007; Yanez-Munoz et al., 2006). Whilst this holds much promise for some disorders that, involve gene transfer to postmitotic tissues, e.g. correction of inherited retinal degeneration, muscle (Apolonia et al., 2007), and possible vaccine development, this approach may not necessarily be particularly useful for haematopoietic conditions that require permanent and sustained correction of the disorder in a population of continuously dividing cells.

An ultimate solution to this problem will most probably be in re-engineering retroviral vectors so that the retroviral insertion takes place only at the specifically desired sites of the host cell chromosome and that the transgene is expressed at normal levels in a tissue-specific manner.

6.2 Final Conclusions

In this study, we have described the generation and testing of a series of enhancer deleted Δ-LTR retroviral vectors that attempted to combine high titre and tissue-specific transcriptional activity to provided highly efficient restoration of superoxide generation. These vectors were tested in both myeloid cell lines *in vitro* and *in vivo* in a mouse model of autosomal recessive CGD.

The Δ-LTR vectors gave acceptable levels of gene transfer to mouse bone marrow cells. Evidence for a slight preferential expression in myeloid cells was obtained with all the vectors studied (Figure 29 and Table 4). Nitroblue tetrazolium assay of superoxide generation in mouse bone marrow derived haematopoietic colonies revealed that transduction with Δ-LTR vectors could restore functional NADPH oxidase to cells from these animals. Superoxide assay of peripheral blood confirmed that, although relatively low numbers of cells were transduced, the Δ-LTR vector was capable of reconstituting very high levels of oxidase activity, comparable to that obtained from normal cells.

We surmise that, in the absence of a non-tissue-specific enhancer, the C/EBP binding site in the Δ-LTR vector may be mediating transcription in myeloid cells, albeit at somewhat reduced levels of expression compared with an intact LTR. In support of this interpretation is the observation that slightly larger deletions which also encompass the viral promoter, so-called SIN vectors (Delviks et al., 1997; Iwakuma et al., 1999; Naviaux et al., 1996; Yu et al., 1986), seem to render the LTR transcriptionally inert.

Interestingly, although C/EBP factors have the correct tissue specificity for expression of $p47^{phox}$, their kinetics differ quite significantly and no C/EBP binding sites have been identified in the $p47^{phox}$ promoter itself (Li et al., 1997; Marden et al., 2003a; Marden et al., 2003b). For example, maximal expression of C/EBPα in neutrophils develops prior to terminal differentiation, declining at a time when $p47^{phox}$ is normally accumulating. Despite this difference, it does not appear to affect accumulation of $p47^{phox}$ in Δ-LTR vector-transduced cells profoundly. On the other hand, it might account for the poorer representation of transduced macrophages in bone marrow we observed in the FACs analysis (Figure 29 and Table 4) compared with the WT-LTR vector.

It is notable that an alternative SIN-vector approach to the development of vectors for CGD retroviral gene therapy has also been described (Roesler *et al.*, 2002). This system has displayed impressive levels of correction in CGD patient cells used to transplant NOD/SCID mice. Whilst it possesses many, though not all, of the virtues of our own vector system, as it was based on a lentiviral vector backbone it has potential advantages for applications requiring transduction of quiescent cells. HIV-based lentiviral vectors

continue to show much promise but also have significant additional safety concerns of their own.

Unlike conventional retroviral vectors, which require cell division to facilitate integration, lentiviral vectors are promising tools for the development of gene therapy since they can infect and transduce efficiently both quiescent and dividing target cells (Delenda, 2004). Hence, lentiviral vectors are particularly attractive vehicles for gene delivery to haematopoietic stem cells, since these cells tend to be quiescent and are therefore difficult targets for vectors that require dividing cells. They also can provide higher levels of gene expression through the incorporation of enhancing regulatory elements, like the woodchuck post-transcriptional regulatory element (WPRE), which has been shown to have significant beneficial effects on transgene expression and viral titer in both gamma-retroviruses and lentiviruses (Hlavaty et al., 2005; Werner et al., 2004; Zufferey et al., 1999).

However, the major limitation of using lentiviral vectors in clinical trial are the safety concerns related to their HIV origin and more recently a propensity to induce liver tumours in mice (Kingsman et al., 2005; Themis et al., 2005).

As the potential benefits of the use of gamma-retroviral vectors still outweigh the risks, it seems worth exploring methodologies to improve their safety profile without drastically affecting their virtues.

It seems fair to say that the general availability of enhancer-deleted or SIN vectors, lacking powerful enhancers, hereby a reduced mutagenic potential and with improved tissue specificity through an endogenous promoter, should enable the development of materially safer retroviral gene therapy protocols than those currently in clinical usage. Hence, the Δ-LTR vector described here could provide the basis for a new generation of retroviral vectors with improved safety.

It is hoped that with these small but consistent technical advances, gene therapy will in the near future become a routine clinical reality. As Anderson has pointed out (Anderson, 2000): "Gene Therapy will succeed with time. And it is important that it does succeed, because no other area of medicine holds as much promise for providing cures for the many devastating diseases that now ravage humankind".

7 References

Reference List

Aaronson,S.A. and Todaro,G.J. (1968). Development of 3T3-like lines from Balb-c mouse embryo cultures: transformation susceptibility to SV40. *J. Cell Physiol* **72,** 141-148.

Abo,A., Boyhan,A., West,I., Thrasher,A.J., and Segal,A.W. (1992). Reconstitution of neutrophil NADPH oxidase activity in the cell-free system by four components: p67-phox, p47-phox, p21rac1, and cytochrome b-245. *J. Biol. Chem.* **267,** 16767-16770.

Abo,A. and Pick,E. (1991). Purification and characterization of a third cytosolic component of the superoxide-generating NADPH oxidase of macrophages. *J. Biol. Chem.* **266,** 23577-23585.

Abo,A., Pick,E., Hall,A., Totty,N., Teahan,C.G., and Segal,A.W. (1991). Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature* **353,** 668-670.

Abo,A., Webb,M.R., Grogan,A., and Segal,A.W. (1994). Activation of NADPH oxidase involves the dissociation of p21rac from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane. *Biochem. J.* **298 Pt 3,** 585-591.

Acland,G.M., Aguirre,G.D., Ray,J., Zhang,Q., Aleman,T.S., Cideciyan,A.V., Pearce-Kelling,S.E., Anand,V., Zeng,Y., Maguire,A.M. et al. (2001). Gene therapy restores vision in a canine model of childhood blindness. *Nat. Genet.* **28,** 92-95.

Ahmed,S., Prigmore,E., Govind,S., Veryard,C., Kozma,R., Wientjes,F.B., Segal,A.W., and Lim,L. (1998). Cryptic Rac-binding and p21(Cdc42Hs/Rac)-activated kinase phosphorylation sites of NADPH oxidase component p67(phox). *J. Biol. Chem.* **273,** 15693-15701.

Aiuti,A., Slavin,S., Aker,M., Ficara,F., Deola,S., Mortellaro,A., Morecki,S., Andolfi,G., Tabucchi,A., Carlucci,F. et al. (2002). Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* **296,** 2410-2413.

Akgun,E., Ziegler,M., and Grez,M. (1991). Determinants of retrovirus gene expression in embryonal carcinoma cells. *J. Virol.* **65,** 382-388.

Amado,R.G. and Chen,I.S. (1999). Lentiviral vectors--the promise of gene therapy within reach? *Science* **285,** 674-676.

Ambruso,D.R., Knall,C., Abell,A.N., Panepinto,J., Kurkchubasche,A., Thurman,G., Gonzalez-Aller,C., Hiester,A., deBoer,M., Harbeck,R.J. et al. (2000). Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. *Proc. Natl. Acad. Sci. U. S. A* **97,** 4654-4659.

Anderson,K.L., Smith,K.A., Conners,K., McKercher,S.R., Maki,R.A., and Torbett,B.E. (1998a). Myeloid development is selectively disrupted in PU.1 null mice. *Blood* **91,** 3702-3710.

Anderson,K.L., Smith,K.A., Pio,F., Torbett,B.E., and Maki,R.A. (1998b). Neutrophils deficient in PU.1 do not terminally differentiate or become functionally competent. *Blood* **92,** 1576-1585.

Anderson,W.F. (2000). Gene therapy. The best of times, the worst of times. *Science* **288,** 627-629.

Anson,D.S., McIntyre,C., Thomas,B., Koldej,R., Ranieri,E., Roberts,A., Clements,P.R., Dunning,K., and Byers,S. (2007). Lentiviral-mediated gene correction of mucopolysaccharidosis type IIIA. *Genet. Vaccines. Ther.* **5,** 1.

Apolonia,L., Waddington,S.N., Fernandes,C., Ward,N.J., Bouma,G., Blundell,M.P., Thrasher,A.J., Collins,M.K., and Philpott,N.J. (2007). Stable gene transfer to muscle using non-integrating lentiviral vectors. *Mol. Ther.* **15,** 1947-1954.

Armesilla,A.L., Calvo,D., and Vega,M.A. (1996). Structural and functional characterization of the human CD36 gene promoter: identification of a proximal PEBP2/CBF site. *J. Biol. Chem.* **271,** 7781-7787.

References

Artelt,P., Morelle,C., Ausmeier,M., Fitzek,M., and Hauser,H. (1988). Vectors for efficient expression in mammalian fibroblastoid, myeloid and lymphoid cells via transfection or infection. *Gene* **68,** 213-219.

Babior,B.M. (1999). NADPH oxidase: an update. *Blood* **93,** 1464-1476.

Babior,B.M. (2004). NADPH oxidase. *Curr. Opin. Immunol.* **16,** 42-47.

Babior,B.M., Kipnes,R.S., and Curnutte,J.T. (1973). Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest* **52,** 741-744.

Baltimore,D. (1970). RNA-dependent DNA polymerase in virions of RNA tumour viruses. *Nature* **226,** 1209-1211.

Banfi,B., Schrenzel,J., Nusse,O., Lew,D.P., Ligeti,E., Krause,K.H., and Demaurex,N. (1999). A novel H(+) conductance in eosinophils: unique characteristics and absence in chronic granulomatous disease. *J. Exp. Med.* **190,** 183-194.

Barese,C.N., Goebel,W.S., and Dinauer,M.C. (2004). Gene therapy for chronic granulomatous disease. *Expert. Opin. Biol. Ther.* **4,** 1423-1434.

Bauer-TR,J., Osborne,W.R., Kwok,W.W., and Hickstein,D.D. (1994). Expression from leukocyte integrin promoters in retroviral vectors. *Hum. Gene Ther.* **5,** 709-716.

Baum,C., Dullmann,J., Li,Z., Fehse,B., Meyer,J., Williams,D.A., and von,K.C. (2003). Side effects of retroviral gene transfer into hematopoietic stem cells. *Blood* **101,** 2099-2114.

Baum,C., Schambach,A., Bohne,J., and Galla,M. (2006). Retrovirus vectors: toward the plentivirus? *Mol. Ther.* **13,** 1050-1063.

Bemiller,L.S., Roberts,D.H., Starko,K.M., and Curnutte,J.T. (1995). Safety and effectiveness of long-term interferon gamma therapy in patients with chronic granulomatous disease. *Blood Cells Mol. Dis.* **21,** 239-247.

Berendes,H., Bridges,R.A., and Good,R.A. (1957). A fatal granulomatosus of childhood: the clinical study of a new syndrome. *Minn. Med.* **40,** 309-312.

Bielorai,B., Toren,A., Wolach,B., Mandel,M., Golan,H., Neumann,Y., Kaplinisky,C., Weintraub,M., Keller,N., Amariglio,N. et al. (2000). Successful treatment of invasive aspergillosis in chronic granulomatous disease by granulocyte transfusions followed by peripheral blood stem cell transplantation. *Bone Marrow Transplant.* **26,** 1025-1028.

Bjorgvinsdottir,H., Ding,C., Pech,N., Gifford,M.A., Li,L.L., and Dinauer,M.C. (1997). Retroviral-mediated gene transfer of gp91phox into bone marrow cells rescues defect in host defense against Aspergillus fumigatus in murine X-linked chronic granulomatous disease. *Blood* **89,** 41-48.

Blaese,R.M., Culver,K.W., Miller,A.D., Carter,C.S., Fleisher,T., Clerici,M., Shearer,G., Chang,L., Chiang,Y., Tolstoshev,P. et al. (1995). T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science* **270,** 475-480.

Bokoch,G.M. (1995). Chemoattractant signaling and leukocyte activation. *Blood* **86,** 1649-1660.

Bordignon,C., Notarangelo,L.D., Nobili,N., Ferrari,G., Casorati,G., Panina,P., Mazzolari,E., Maggioni,D., Rossi,C., Servida,P. et al. (1995). Gene therapy in peripheral blood lymphocytes and bone marrow for ADA- immunodeficient patients. *Science* **270,** 470-475.

Borregaard,N., Theilgaard-Monch,K., Sorensen,O.E., and Cowland,J.B. (2001). Regulation of human neutrophil granule protein expression. *Curr. Opin. Hematol.* **8,** 23-27.

Brenner,M.K. (1996). Gene transfer to hematopoietic cells. *N. Engl. J. Med.* **335,** 337-339.

Britos-Bray,M. and Friedman,A.D. (1997). Core binding factor cannot synergistically activate the myeloperoxidase proximal enhancer in immature myeloid cells without c-Myb. *Mol. Cell Biol.* **17,** 5127-5135.

Bu-Ghanim,H.N., Segal,A.W., Keep,N.H., and Casimir,C.M. (1995). Molecular analysis in three cases of X91- variant chronic granulomatous disease. *Blood* **86,** 3575-3582.

Bukrinsky,M.I., Haggerty,S., Dempsey,M.P., Sharova,N., Adzhubel,A., Spitz,L., Lewis,P.,

References

Goldfarb,D., Emerman,M., and Stevenson,M. (1993). A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature* **365,** 666-669.

Casimir,C., Chetty,M., Bohler,M.C., Garcia,R., Fischer,A., Griscelli,C., Johnson,B., and Segal,A.W. (1992). Identification of the defective NADPH-oxidase component in chronic granulomatous disease: a study of 57 European families. *Eur. J. Clin. Invest* **22,** 403-406.

Casimir,C.M., Bu-Ghanim,H.N., Rodaway,A.R., Bentley,D.L., Rowe,P., and Segal,A.W. (1991). Autosomal recessive chronic granulomatous disease caused by deletion at a dinucleotide repeat. *Proc. Natl. Acad. Sci. U. S. A* **88,** 2753-2757.

Casimir,C.M. and Teahan,C.G. (1994). The respiratory burst of neutrophils and its deficiency. In *(Handbook of Immunopharmacology)*. pp. 27-54. Academic Press Limited Inc41.

Cavazzana-Calvo,M., Hacein-Bey,S., de Saint,B.G., Gross,F., Yvon,E., Nusbaum,P., Selz,F., Hue,C., Certain,S., Casanova,J.L. et al. (2000a). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288,** 669-672.

Cavazzana-Calvo,M., Hacein-Bey,S., de Saint,B.G., Gross,F., Yvon,E., Nusbaum,P., Selz,F., Hue,C., Certain,S., Casanova,J.L. et al. (2000b). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease [see comments]. *Science* **288,** 669-672.

Cavazzana-Calvo,M., Hacein-Bey,S., Yates,F., de Villartay,J.P., Le,D.F., and Fischer,A. (2001). Gene therapy of severe combined immunodeficiencies. *J. Gene Med.* **3,** 201-206.

Challita,P.M. and Kohn,D.B. (1994). Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2567-2571.

Challita,P.M., Skelton,D., el Khoueiry,A., Yu,X.J., Weinberg,K., and Kohn,D.B. (1995). Multiple modifications in cis elements of the long terminal repeat of retroviral vectors lead to increased expression and decreased DNA methylation in embryonic carcinoma cells. *J. Virol.* **69,** 748-755.

Chan,Y.J., Chiou,C.J., Huang,Q., and Hayward,G.S. (1996). Synergistic interactions between overlapping binding sites for the serum response factor and ELK-1 proteins mediate both basal enhancement and phorbol ester responsiveness of primate cytomegalovirus major immediate-early promoters in monocyte and T-lymphocyte cell types. *J. Virol.* **70,** 8590-8605.

Chanock,S.J., Roesler,J., Zhan,S., Hopkins,P., Lee,P., Barrett,D.T., Christensen,B.L., Curnutte,J.T., and Gorlach,A. (2000). Genomic structure of the human p47-phox (NCF1) gene. *Blood Cells Mol. Dis.* **26,** 37-46.

Chen,H., Ray,G.D., Zhang,P., Hetherington,C.J., Gonzalez,D.A., Zhang,D.E., Moreau,G.F., and Tenen,D.G. (1995a). PU.1 (Spi-1) autoregulates its expression in myeloid cells. *Oncogene* **11,** 1549-1560.

Chen,H.M., Pahl,H.L., Scheibe,R.J., Zhang,D.E., and Tenen,D.G. (1993). The Sp1 transcription factor binds the CD11b promoter specifically in myeloid cells in vivo and is essential for myeloid-specific promoter activity. *J. Biol. Chem.* **268,** 8230-8239.

Chen,H.M., Zhang,P., Voso,M.T., Hohaus,S., Gonzalez,D.A., Glass,C.K., Zhang,D.E., and Tenen,D.G. (1995b). Neutrophils and monocytes express high levels of PU.1 (Spi-1) but not Spi-B. *Blood* **85,** 2918-2928.

Chin,T.W., Stiehm,E.R., Falloon,J., and Gallin,J.I. (1987). Corticosteroids in treatment of obstructive lesions of chronic granulomatous disease. *J. Pediatr.* **111,** 349-352.

Clark,R.A. (1999). Activation of the neutrophil respiratory burst oxidase. *J. Infect. Dis.* **179 Suppl 2,** S309-S317.

Clark,R.A., Malech,H.L., Gallin,J.I., Nunoi,H., Volpp,B.D., Pearson,D.W., Nauseef,W.M., and Curnutte,J.T. (1989). Genetic variants of chronic granulomatous disease: prevalence of deficiencies of two cytosolic components of the NADPH oxidase system. *N. Engl. J. Med.* **321,** 647-652.

References

Clarke,S. and Gordon,S. (1998). Myeloid-specific gene expression. *J. Leukoc. Biol.* **63,** 153-168.

Cockcroft,S., Baldwin,J.M., and Allan,D. (1984). The Ca2+-activated polyphosphoinositide phosphodiesterase of human and rabbit neutrophil membranes. *Biochem. J.* **221,** 477-482.

Cockcroft,S. and Gomperts,B.D. (1985). Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. *Nature* **314,** 534-536.

Coffin,J.M., Hughes,S.H., and Varmus,H.E. (1997). *Retroviruses.* New York: Cold Spring Harbor Laboratory Press.

Cohen,M.S. (1994). Molecular events in the activation of human neutrophils for microbial killing. *Clin. Infect. Dis.* **18 Suppl 2,** S170-S179.

Collins,S.J., Gallo,R.C., and Gallagher,R.E. (1977). Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature* **270,** 347-349.

Cone,R.D. and Mulligan,R.C. (1984). High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. *Proc. Natl. Acad. Sci. U. S. A* **81,** 6349-6353.

Conrad,C.K., Allen,S.S., Afione,S.A., Reynolds,T.C., Beck,S.E., Fee-Maki,M., Barrazza-Ortiz,X., Adams,R., Askin,F.B., Carter,B.J. et al. (1996). Safety of single-dose administration of an adeno-associated virus (AAV)-CFTR vector in the primate lung. *Gene Ther.* **3,** 658-668.

Cormack,B.P., Valdivia,R.H., and Falkow,S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173,** 33-38.

Corneliussen,B., Thornell,A., Hallberg,B., and Grundstrom,T. (1991). Helix-loop-helix transcriptional activators bind to a sequence in glucocorticoid response elements of retrovirus enhancers. *J. Virol.* **65,** 6084-6093.

Cornetta,K., Morgan,R.A., and Anderson,W.F. (1991). Safety issues related to retroviral-mediated gene transfer in humans. *Hum. Gene Ther.* **2,** 5-14.

Cosset,F.L., Takeuchi,Y., Battini,J.L., Weiss,R.A., and Collins,M.K. (1995). High-titer packaging cells producing recombinant retroviruses resistant to human serum. *J. Virol.* **69,** 7430-7436.

Cross,A.R. (2000). p40(phox) Participates in the activation of NADPH oxidase by increasing the affinity of p47(phox) for flavocytochrome b(558). *Biochem. J.* **349,** 113-117.

Crystal,R.G., McElvaney,N.G., Rosenfeld,M.A., Chu,C.S., Mastrangeli,A., Hay,J.G., Brody,S.L., Jaffe,H.A., Eissa,N.T., and Danel,C. (1994). Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. *Nat. Genet.* **8,** 42-51.

Curnutte,J.T. (1993). Chronic granulomatous disease: the solving of a clinical riddle at the molecular level. *Clin. Immunol. Immunopathol.* **67,** S2-15.

Curnutte,J.T. and Babior,B.M. (1987). Chronic granulomatous disease. *Adv. Hum. Genet.* **16,** 229-297.

De Leo,F.R., Ulman,K.V., Davis,A.R., Jutila,K.L., and Quinn,M.T. (1996). Assembly of the human neutrophil NADPH oxidase involves binding of p67phox and flavocytochrome b to a common functional domain in p47phox. *J. Biol. Chem.* **271,** 17013-17020.

de,M., I, Adams,A.G., Sokolic,R.A., Malech,H.L., and Leto,T.L. (1996). Multiple SH3 domain interactions regulate NADPH oxidase assembly in whole cells. *EMBO J.* **15,** 1211-1220.

de,M., I, Garrett,M.C., Adams,A.G., and Leto,T.L. (1994). Role of p67-phox SH3 domains in assembly of the NADPH oxidase system. *J. Biol. Chem.* **269,** 16326-16332.

de,M., I, Homayounpour,N., and Leto,T.L. (1997). Specificity of p47phox SH3 domain interactions in NADPH oxidase assembly and activation. *Mol. Cell Biol.* **17,** 2177-2185.

DeFranco,D. and Yamamoto,K.R. (1986). Two different factors act separately or together to specify functionally distinct activities at a single

transcriptional enhancer. *Mol. Cell Biol.* **6,** 993-1001.

Del,G., I, Iori,A.P., Mengarelli,A., Testi,A.M., Romano,A., Cerretti,R., Macri,F., Iacobini,M., and Arcese,W. (2003). Allogeneic stem cell transplant from HLA-identical sibling for chronic granulomatous disease and review of the literature. *Ann. Hematol.* **82,** 189-192.

Delenda,C. (2004). Lentiviral vectors: optimization of packaging, transduction and gene expression. *J. Gene Med.* **6 Suppl 1,** S125-S138.

DeLeo,F.R., Allen,L.A., Apicella,M., and Nauseef,W.M. (1999). NADPH oxidase activation and assembly during phagocytosis. *J. Immunol.* **163,** 6732-6740.

DeLeo,F.R., Nauseef,W.M., Jesaitis,A.J., Burritt,J.B., Clark,R.A., and Quinn,M.T. (1995a). A domain of p47phox that interacts with human neutrophil flavocytochrome b558. *J. Biol. Chem.* **270,** 26246-26251.

DeLeo,F.R., Yu,L., Burritt,J.B., Loetterle,L.R., Bond,C.W., Jesaitis,A.J., and Quinn,M.T. (1995b). Mapping sites of interaction of p47-phox and flavocytochrome b with random-sequence peptide phage display libraries. *Proc. Natl. Acad. Sci. U. S. A* **92,** 7110-7114.

Delviks,K.A., Hu,W.S., and Pathak,V.K. (1997). Psi- vectors: murine leukemia virus-based self-inactivating and self-activating retroviral vectors. *J. Virol.* **71,** 6218-6224.

Dexter,T.M. and Heyworth,C.M. (1994). Growth factors and the molecular control of haematopoiesis. *Eur. J. Clin. Microbiol. Infect. Dis.* **13 Suppl 2,** S3-S8.

Diaz,R.M., Eisen,T., Hart,I.R., and Vile,R.G. (1998). Exchange of viral promoter/enhancer elements with heterologous regulatory sequences generates targeted hybrid long terminal repeat vectors for gene therapy of melanoma. *J. Virol.* **72,** 789-795.

Diekmann,D., Abo,A., Johnston,C., Segal,A.W., and Hall,A. (1994). Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity. *Science* **265,** 531-533.

Dinauer,M.C. (2005). Chronic granulomatous disease and other disorders of phagocyte function. *Hematology. (Am. Soc. Hematol. Educ. Program.)* 89-95.

Dinauer,M.C., Lekstrom-Himes,J.A., and Dale,D.C. (2000). Inherited Neutrophil Disorders: Molecular Basis and New Therapies. *Hematology. (Am. Soc. Hematol. Educ. Program.)* 303-318.

Dinauer,M.C., Li,L.L., ttir,H., Ding,C., and Pech,N. (1999). Long-Term Correction of Phagocyte NADPH Oxidase Activity by Retroviral- Mediated Gene Transfer in Murine X-Linked Chronic Granulomatous Disease. *Blood* **94,** 914-922.

Dunbar,C.E. (1996). Gene transfer to hematopoietic stem cells: implications for gene therapy of human disease. *Annu. Rev. Med.* **47,** 11-20.

Dusi,S., Donini,M., and Rossi,F. (1996). Mechanisms of NADPH oxidase activation: translocation of p40phox, Rac1 and Rac2 from the cytosol to the membranes in human neutrophils lacking p47phox or p67phox. *Biochem. J.* **314 (Pt 2),** 409-412.

Dusi,S. and Rossi,F. (1993). Activation of NADPH oxidase of human neutrophils involves the phosphorylation and the translocation of cytosolic p67phox. *Biochem. J.* **296 (Pt 2),** 367-371.

Dynan,W.S., Saffer,J.D., Lee,W.S., and Tjian,R. (1985). Transcription factor Sp1 recognizes promoter sequences from the monkey genome that are simian virus 40 promoter. *Proc. Natl. Acad. Sci. U. S. A* **82,** 4915-4919.

Dziennis,S., Van Etten,R.A., Pahl,H.L., Morris,D.L., Rothstein,T.L., Blosch,C.M., Perlmutter,R.M., and Tenen,D.G. (1995). The CD11b promoter directs high-level expression of reporter genes in macrophages in transgenic mice [published erratum appears in Blood 1995 Apr 1;85(7):1983]. *Blood* **85,** 319-329.

Edelstein,M.L., Abedi,M.R., Wixon,J., and Edelstein,R.M. (2004). Gene therapy clinical trials worldwide 1989-2004-an overview. *J. Gene Med.* **6,** 597-602.

References

Eichbaum,Q.G., Iyer,R., Raveh,D.P., Mathieu,C., and Ezekowitz (1994). Restriction of interferon gamma responsiveness and basal expression of the myeloid human Fc gamma R1b gene is mediated by a functional PU.1 site and a transcription initiator consensus. *J. Exp. Med.* **179,** 1985-1996.

Emerman,M. and Temin,H.M. (1984). Genes with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism. *Cell* **39,** 449-467.

Emerman,M. and Temin,H.M. (1986a). Comparison of promoter suppression in avian and murine retrovirus vectors. *Nucleic Acids Res.* **14,** 9381-9396.

Emerman,M. and Temin,H.M. (1986b). Quantitative analysis of gene suppression in integrated retrovirus vectors. *Mol. Cell Biol.* **6,** 792-800.

Emili,A., Greenblatt,J., and Ingles,C.J. (1994). Species-specific interaction of the glutamine-rich activation domains of Sp1 with the TATA box-binding protein. *Mol. Cell Biol.* **14,** 1582-1593.

Emmendorffer,A., Hecht,M., Lohmann-Matthes,M.L., and Roesler,J. (1990). A fast and easy method to determine the production of reactive oxygen intermediates by human and murine phagocytes using dihydrorhodamine 123. *J. Immunol. Methods* **131,** 269-275.

Emmendorffer,A., Nakamura,M., Rothe,G., Spiekermann,K., Lohmann-Matthes,M.L., and Roesler,J. (1994). Evaluation of flow cytometric methods for diagnosis of chronic granulomatous disease variants under routine laboratory conditions. *Cytometry* **18,** 147-155.

Evans,L.H., Morrison,R.P., Malik,F.G., Portis,J., and Britt,W.J. (1990). A neutralizable epitope common to the envelope glycoproteins of ecotropic, polytropic, xenotropic, and amphotropic murine leukemia viruses. *J. Virol.* **64,** 6176-6183.

Ezekowitz,R.A., Dinauer,M.C., Jaffe,H.S., Orkin,S.H., and Newburger,P.E. (1988). Partial correction of the phagocyte defect in patients with X-linked chronic granulomatous disease by subcutaneous interferon gamma. *N. Engl. J. Med.* **319,** 146-151.

Faust,L.R., el,B.J., Babior,B.M., and Chanock,S.J. (1995). The phosphorylation targets of p47phox, a subunit of the respiratory burst oxidase. Functions of the individual target serines as evaluated by site-directed mutagenesis. *J. Clin. Invest* **96,** 1499-1505.

Feinman,R., Qiu,W.Q., Pearse,R.N., Nikolajczyk,B.S., Sen,R., Sheffery,M., and Ravetch,J.V. (1994). PU.1 and an HLH family member contribute to the myeloid-specific transcription of the Fc gamma RIIIA promoter. *EMBO J.* **13,** 3852-3860.

Felgner,J.H., Kumar,R., Sridhar,C.N., Wheeler,C.J., Tsai,Y.J., Border,R., Ramsey,P., Martin,M., and Felgner,P.L. (1994). Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Biol. Chem.* **269,** 2550-2561.

Felgner,P.L., Gadek,T.R., Holm,M., Roman,R., Chan,H.W., Wenz,M., Northrop,J.P., Ringold,G.M., and Danielsen,M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U. S. A* **84,** 7413-7417.

Fisher,K.J., Jooss,K., Alston,J., Yang,Y., Haecker,S.E., High,K., Pathak,R., Raper,S.E., and Wilson,J.M. (1997). Recombinant adeno-associated virus for muscle directed gene therapy. *Nat. Med.* **3,** 306-312.

Flanagan,J.R., Becker,K.G., Ennist,D.L., Gleason,S.L., Driggers,P.H., Levi,B.Z., Appella,E., and Ozato,K. (1992). Cloning of a negative transcription factor that binds to the upstream conserved region of Moloney murine leukemia virus. *Mol. Cell Biol.* **12,** 38-44.

Flotte,T., Carter,B., Conrad,C., Guggino,W., Reynolds,T., Rosenstein,B., Taylor,G., Walden,S., and Wetzel,R. (1996). A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease. *Hum. Gene Ther.* **7,** 1145-1159.

Flotte,T.R., Schwiebert,E.M., Zeitlin,P.L., Carter,B.J., and Guggino,W.B. (2005). Correlation between DNA transfer and cystic fibrosis airway epithelial cell correction after recombinant adeno-associated virus serotype 2 gene therapy. *Hum. Gene Ther.* **16,** 921-928.

References

Flotte,T.R., Zeitlin,P.L., Reynolds,T.C., Heald,A.E., Pedersen,P., Beck,S., Conrad,C.K., Brass-Ernst,L., Humphries,M., Sullivan,K. et al. (2003). Phase I trial of intranasal and endobronchial administration of a recombinant adeno-associated virus serotype 2 (rAAV2)-CFTR vector in adult cystic fibrosis patients: a two-part clinical study. *Hum. Gene Ther.* **14,** 1079-1088.

Foster,C.B., Lehrnbecher,T., Mol,F., Steinberg,S.M., Venzon,D.J., Walsh,T.J., Noack,D., Rae,J., Winkelstein,J.A., Curnutte,J.T. et al. (1998). Host defense molecule polymorphisms influence the risk for immune- mediated complications in chronic granulomatous disease. *J. Clin. Invest* **102,** 2146-2155.

Fouret,P., du Bois,R.M., Bernaudin,J.F., Takahashi,H., Ferrans,V.J., and Crystal,R.G. (1989). Expression of the neutrophil elastase gene during human bone marrow cell differentiation. *J. Exp. Med.* **169,** 833-845.

Friedman,A.D. (2002). Transcriptional regulation of granulocyte and monocyte development. *Oncogene* **21,** 3377-3390.

Friedman,A.D., Keefer,J.R., Kummalue,T., Liu,H., Wang,Q.F., and Cleaves,R. (2003). Regulation of granulocyte and monocyte differentiation by CCAAT/enhancer binding protein alpha. *Blood Cells Mol. Dis.* **31,** 338-341.

Fuchs,A., Dagher,M.C., Faure,J., and Vignais,P.V. (1996). Topological organization of the cytosolic activating complex of the superoxide-generating NADPH-oxidase. Pinpointing the sites of interaction between p47phoz, p67phox and p40phox using the two-hybrid system. *Biochim. Biophys. Acta* **1312,** 39-47.

Fuchs,A., Dagher,M.C., and Vignais,P.V. (1995). Mapping the domains of interaction of p40phox with both p47phox and p67phox of the neutrophil oxidase complex using the two-hybrid system. *J. Biol. Chem.* **270,** 5695-5697.

Gallagher,R., Collins,S., Trujillo,J., McCredie,K., Ahearn,M., Tsai,S., Metzgar,R., Aulakh,G., Ting,R., Ruscetti,F. et al. (1979). Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. *Blood* **54,** 713-733.

Gaspar,H.B., Bjorkegren,E., Parsley,K., Gilmour,K.C., King,D., Sinclair,J., Zhang,F., Giannakopoulos,A., Adams,S., Fairbanks,L.D. et al. (2006). Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. *Mol. Ther.* **14,** 505-513.

Gilmour,K.C., Cranston,T., Jones,A., Davies,E.G., Goldblatt,D., Thrasher,A., Kinnon,C., Nichols,K.E., and Gaspar,H.B. (2000). Diagnosis of X-linked lymphoproliferative disease by analysis of SLAM-associated protein expression. *Eur. J. Immunol.* **30,** 1691-1697.

Goebel,W.S. and Dinauer,M.C. (2003). Gene therapy for chronic granulomatous disease. *Acta Haematol.* **110,** 86-92.

Goldblatt,D. and Thrasher,A.J. (2000). Chronic granulomatous disease. *Clin. Exp. Immunol.* **122,** 1-9.

Gorlach,A., Lee,P.L., Roesler,J., Hopkins,P.J., Christensen,B., Green,E.D., Chanock,S.J., and Curnutte,J.T. (1997). A p47-phox pseudogene carries the most common mutation causing p47-phox- deficient chronic granulomatous disease. *J. Clin. Invest* **100,** 1907-1918.

Gorman,C.M., Rigby,P.W., and Lane,D.P. (1985). Negative regulation of viral enhancers in undifferentiated embryonic stem cells. *Cell* **42,** 519-526.

Goudemand,J., Anssens,R., mas-Marsalet,Y., Farriaux,J.P., and Fontaine,G. (1976). [Attempt to treat a case of chronic familial granulomatous disease by allogenic bone marrow transplantation]. *Arch. Fr. Pediatr.* **33,** 121-129.

Graham,F.L., Smiley,J., Russell,W.C., and Nairn,R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36,** 59-74.

Graham,F.L. and van der Eb,A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52,** 456-467.

Graves,B.J., Johnson,P.F., and McKnight,S.L. (1986). Homologous recognition of a promoter

domain common to the MSV LTR and the HSV tk gene. *Cell* **44,** 565-576.

Greer,P., Maltby,V., Rossant,J., Bernstein,A., and Pawson,T. (1990). Myeloid expression of the human c-fps/fes proto-oncogene in transgenic mice. *Mol. Cell Biol.* **10,** 2521-2527.

Grez,M., Akgun,E., Hilberg,F., and Ostertag,W. (1990). Embryonic stem cell virus, a recombinant murine retrovirus with expression in embryonic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9202-9206.

Grez,M., Becker,S., Saulnier,S., Knoss,H., Ott,M.G., Maurer,A., Dinauer,M.C., Hoelzer,D., Seger,R., and Hossle,J.P. (2000). Gene therapy of chronic granulomatous disease. *Bone Marrow Transplant.* **25 Suppl 2,** S99-104.

Grignani,F., Kinsella,T., Mencarelli,A., Valtieri,M., Riganelli,D., Grignani,F., Lanfrancone,L., Peschle,C., Nolan,G.P., and Pelicci,P.G. (1998). High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. *Cancer Res.* **58,** 14-19.

Grove,M. and Plumb,M. (1993). C/EBP, NF-kappa B, and c-Ets family members and transcriptional regulation of the cell-specific and inducible macrophage inflammatory protein 1 alpha immediate-early gene. *Mol. Cell Biol.* **13,** 5276-5289.

Gunther,C.V. and Graves,B.J. (1994). Identification of ETS domain proteins in murine T lymphocytes that interact with the Moloney murine leukemia virus enhancer. *Mol. Cell Biol.* **14,** 7569-7580.

Hacein-Bey-Abina,S., de Saint,B.G., and Cavazzana-Calvo,M. (2003a). Gene therapy of X-linked severe combined immunodeficiency. *Methods Mol. Biol.* **215,** 247-259.

Hacein-Bey-Abina,S., Fischer,A., and Cavazzana-Calvo,M. (2002a). Gene therapy of X-linked severe combined immunodeficiency. *Int. J. Hematol.* **76,** 295-298.

Hacein-Bey-Abina,S., Le Deist,F., Carlier,F., Bouneaud,C., Hue,C., de Villartay,J.P., Thrasher,A.J., Wulffraat,N., Sorensen,R., Dupuis-Girod,S. et al. (2002b). Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N. Engl. J. Med.* **346,** 1185-1193.

Hacein-Bey-Abina,S., Von Kalle,C., Schmidt,M., Le Deist,F., Wulffraat,N., McIntyre,E., Radford,I., Villeval,J.L., Fraser,C.C., Cavazzana-Calvo,M. et al. (2003b). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* **348,** 255-256.

Hacein-Bey-Abina,S., Von Kalle,C., Schmidt,M., McCormack,M.P., Wulffraat,N., Leboulch,P., Lim,A., Osborne,C.S., Pawliuk,R., Morillon,E. et al. (2003c). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302,** 415-419.

Hagemeier,C., Bannister,A.J., Cook,A., and Kouzarides,T. (1993). The activation domain of transcription factor PU.1 binds the retinoblastoma (RB) protein and the transcription factor TFIID in vitro: RB shows sequence similarity to TFIID and TFIIB. *Proc. Natl. Acad. Sci. U. S. A* **90,** 1580-1584.

Hart,S.L., Arancibia-Carcamo,C.V., Wolfert,M.A., Mailhos,C., O'Reilly,N.J., Ali,R.R., Coutelle,C., George,A.J., Harbottle,R.P., Knight,A.M. et al. (1998). Lipid-mediated enhancement of transfection by a nonviral integrin- targeting vector. *Hum. Gene Ther.* **9,** 575-585.

Hart,S.L., Collins,L., Gustafsson,K., and Fabre,J.W. (1997). Integrin-mediated transfection with peptides containing arginine-glycine-aspartic acid domains. *Gene Ther.* **4,** 1225-1230.

Hart,S.L., Harbottle,R.P., Cooper,R., Miller,A., Williamson,R., and Coutelle,C. (1995). Gene delivery and expression mediated by an integrin-binding peptide [published erratum appears in Gene Ther 1996 Nov;3(11):1032-3]. *Gene Ther.* **2,** 552-554.

Hart,S.L., Knight,A.M., Harbottle,R.P., Mistry,A., Hunger,H.D., Cutler,D.F., Williamson,R., and Coutelle,C. (1994). Cell binding and internalization by filamentous phage displaying a cyclic Arg-Gly-Asp-containing peptide. *J. Biol. Chem.* **269,** 12468-12474.

References

Hauses,M., Tonjes,R.R., and Grez,M. (1998). The transcription factor Sp1 regulates the myeloid-specific expression of the human hematopoietic cell kinase (HCK) gene through binding to two adjacent GC boxes within the HCK promoter-proximal region. *J. Biol. Chem.* **273,** 31844-31852.

Haviernik,P. and Bunting,K.D. (2004). Safety concerns related to hematopoietic stem cell gene transfer using retroviral vectors. *Curr. Gene Ther.* **4,** 263-276.

Henderson,L.M., Banting,G., and Chappell,J.B. (1995). The arachidonate-activable, NADPH oxidase-associated H+ channel. Evidence that gp91-phox functions as an essential part of the channel. *J. Biol. Chem.* **270,** 5909-5916.

Henderson,L.M. and Meech,R.W. (1999). Evidence that the product of the human X-linked CGD gene, gp91-phox, is a voltage-gated H(+) pathway. *J. Gen. Physiol* **114,** 771-786.

Henderson,L.M., Thomas,S., Banting,G., and Chappell,J.B. (1997). The arachidonate-activatable, NADPH oxidase-associated H+ channel is contained within the multi-membrane-spanning N-terminal region of gp91- phox. *Biochem. J.* **325 (Pt 3),** 701-705.

Heydemann,A., Boehmler,J.H., and Simon,M.C. (1997). Expression of two myeloid cell-specific genes requires the novel transcription factor, c-fes expression factor. *J. Biol. Chem.* **272,** 29527-29537.

Heydemann,A., Juang,G., Hennessy,K., Parmacek,M.S., and Simon,M.C. (1996). The myeloid-cell-specific c-fes promoter is regulated by Sp1, PU.1, and a novel transcription factor. *Mol. Cell Biol.* **16,** 1676-1686.

Heydemann,A., Warming,S., Clendenin,C., Sigrist,K., Hjorth,J.P., and Simon,M.C. (2000). A minimal c-fes cassette directs myeloid-specific expression in transgenic mice. *Blood* **96,** 3040-3048.

Heyworth,P.G. and Badwey,J.A. (1990). Continuous phosphorylation of both the 47 and the 49 kDa proteins occurs during superoxide production by neutrophils. *Biochim. Biophys. Acta* **1052,** 299-305.

Heyworth,P.G., Bohl,B.P., Bokoch,G.M., and Curnutte,J.T. (1994). Rac translocates independently of the neutrophil NADPH oxidase components p47phox and p67phox. Evidence for its interaction with flavocytochrome b558. *J. Biol. Chem.* **269,** 30749-30752.

Heyworth,P.G., Cross,A.R., and Curnutte,J.T. (2003). Chronic granulomatous disease. *Curr. Opin. Immunol.* **15,** 578-584.

Heyworth,P.G., Curnutte,J.T., Nauseef,W.M., Volpp,B.D., Pearson,D.W., Rosen,H., and Clark,R.A. (1991). Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox and p67-phox requires interaction between p47-phox and cytochrome b558. *J. Clin. Invest* **87,** 352-356.

Heyworth,P.G., Knaus,U.G., Xu,X., Uhlinger,D.J., Conroy,L., Bokoch,G.M., and Curnutte,J.T. (1993). Requirement for posttranslational processing of Rac GTP-binding proteins for activation of human neutrophil NADPH oxidase. *Mol. Biol. Cell* **4,** 261-269.

Hickstein,D.D., Back,A.L., and Collins,S.J. (1989). Regulation of expression of the CD11b and CD18 subunits of the neutrophil adherence receptor during human myeloid differentiation. *J. Biol. Chem.* **264,** 21812-21817.

Hickstein,D.D., Baker,D.M., Gollahon,K.A., and Back,A.L. (1992). Identification of the promoter of the myelomonocytic leukocyte integrin CD11b. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2105-2109.

Hirt,W., Nebe,T., and Birr,C. (1994). [Phagotest and Bursttest (Phagoburst), test kits for study of phagocyte functions]. *Wien. Klin. Wochenschr.* **106,** 250-252.

Hlavaty,J., Schittmayer,M., Stracke,A., Jandl,G., Knapp,E., Felber,B.K., Salmons,B., Gunzburg,W.H., and Renner,M. (2005). Effect of posttranscriptional regulatory elements on transgene expression and virus production in the context of retrovirus vectors. *Virology* **341,** 1-11.

Hohaus,S., Petrovick,M.S., Voso,M.T., Sun,Z., Zhang,D.E., and Tenen,D.G. (1995). PU.1 (Spi-1) and C/EBP alpha regulate expression of the granulocyte-macrophage colony-stimulating factor receptor alpha gene. *Mol. Cell Biol.* **15,** 5830-5845.

References

Huang,J., Hitt,N.D., and Kleinberg,M.E. (1995). Stoichiometry of p22-phox and gp91-phox in phagocyte cytochrome b558. *Biochemistry* **34,** 16753-16757.

Ito,T., Nakamura,R., Sumimoto,H., Takeshige,K., and Sakaki,Y. (1996). An SH3 domain-mediated interaction between the phagocyte NADPH oxidase factors p40phox and p47phox. *FEBS Lett.* **385,** 229-232.

Iwakuma,T., Cui,Y., and Chang,L.J. (1999). Self-inactivating lentiviral vectors with U3 and U5 modifications. *Virology* **261,** 120-132.

Iwata,M., Nunoi,H., Yamazaki,H., Nakano,T., Niwa,H., Tsuruta,S., Ohga,S., Ohmi,S., Kanegasaki,S., and Matsuda,I. (1994). Homologous dinucleotide (GT or TG) deletion in Japanese patients with chronic granulomatous disease with p47-phox deficiency. *Biochem. Biophys. Res. Commun.* **199,** 1372-1377.

Jackson,S.H., Gallin,J.I., and Holland,S.M. (1995). The p47phox mouse knock-out model of chronic granulomatous disease. *J. Exp. Med.* **182,** 751-758.

Jager,U., Zhao,Y., and Porter,C.D. (1999). Endothelial cell-specific transcriptional targeting from a hybrid long terminal repeat retrovirus vector containing human prepro-endothelin-1 promoter sequences. *J. Virol.* **73,** 9702-9709.

Janson,C., McPhee,S., Bilaniuk,L., Haselgrove,J., Testaiuti,M., Freese,A., Wang,D.J., Shera,D., Hurh,P., Rupin,J. et al. (2002). Clinical protocol. Gene therapy of Canavan disease: AAV-2 vector for neurosurgical delivery of aspartoacylase gene (ASPA) to the human brain. *Hum. Gene Ther.* **13,** 1391-1412.

Jones,I.M. and Morikawa,Y. (1998). The molecular basis of HIV capsid assembly. *Rev. Med. Virol.* **8,** 87-95.

Jones,O.T. (1994). The regulation of superoxide production by the NADPH oxidase of neutrophils and other mammalian cells. *Bioessays* **16,** 919-923.

Joshi,S., Van Brunschot,A., Robson,I., and Bernstein,A. (1990). Efficient replication, integration, and packaging of retroviral vectors with modified long terminal repeats containing the packaging signal. *Nucleic Acids Res.* **18,** 4223-4226.

Kaiser,J. (2003). Gene therapy. Seeking the cause of induced leukemias in X-SCID trial. *Science* **299,** 495.

Kay,M.A., Manno,C.S., Ragni,M.V., Larson,P.J., Couto,L.B., McClelland,A., Glader,B., Chew,A.J., Tai,S.J., Herzog,R.W. et al. (2000). Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat. Genet.* **24,** 257-261.

Kefalas,P., Brown,T.R., Katz,D.R., and Brickell,P.M. (1995). Identification of a phorbol ester responsive region in the myeloid-specific promoter of the c-fgr proto-oncogene. *Biochem. Biophys. Res. Commun.* **209,** 60-65.

Keller,G. (1992). Hematopoietic stem cells. *Curr. Opin. Immunol.* **4,** 133-139.

Kempler,G., Freitag,B., Berwin,B., Nanassy,O., and Barklis,E. (1993). Characterization of the Moloney murine leukemia virus stem cell-specific repressor binding site. *Virology* **193,** 690-699.

Kessler,P.D., Podsakoff,G.M., Chen,X., McQuiston,S.A., Colosi,P.C., Matelis,L.A., Kurtzman,G.J., and Byrne,B.J. (1996). Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc. Natl. Acad. Sci. U. S. A* **93,** 14082-14087.

Khanna-Gupta,A., Zibello,T., Simkevich,C., Rosmarin,A.G., and Berliner,N. (2000). Sp1 and C/EBP are necessary to activate the lactoferrin gene promoter during myeloid differentiation. *Blood* **95,** 3734-3741.

Kingsman,S.M., Mitrophanous,K., and Olsen,J.C. (2005). Potential oncogene activity of the woodchuck hepatitis post-transcriptional regulatory element (WPRE). *Gene Ther.* **12,** 3-4.

Kinsella,T.M. and Nolan,G.P. (1996). Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum. Gene Ther.* **7,** 1405-1413.

Kitamura,T., Tojo,A., Kuwaki,T., Chiba,S., Miyazono,K., Urabe,A., and Takaku,F.

(1989). Identification and analysis of human erythropoietin receptors on a factor-dependent cell line, TF-1. *Blood* **73,** 375-380.

Klemsz,M.J., McKercher,S.R., Celada,A., Van,B.C., and Maki,R.A. (1990). The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. *Cell* **61,** 113-124.

Kohn,D.B. (1997). Gene therapy for haematopoietic and lymphoid disorders. *Clin. Exp. Immunol.* **107 Suppl 1,** 54-57.

Kohn,D.B., Sadelain,M., and Glorioso,J.C. (2003). Occurrence of leukaemia following gene therapy of X-linked SCID. *Nat. Rev. Cancer* **3,** 477-488.

Kominato,Y., Galson,D., Waterman,W.R., Webb,A.C., and Auron,P.E. (1995). Monocyte expression of the human prointerleukin 1 beta gene (IL1B) is dependent on promoter sequences which bind the hematopoietic transcription factor Spi-1/PU.1. *Mol. Cell Biol.* **15,** 58-68.

Kraunus,J., Schaumann,D.H., Meyer,J., Modlich,U., Fehse,B., Brandenburg,G., von,L.D., Klump,H., Schambach,A., Bohne,J. et al. (2004). Self-inactivating retroviral vectors with improved RNA processing. *Gene Ther.* **11,** 1568-1578.

Kume,A. and Dinauer,M.C. (2000). Gene therapy for chronic granulomatous disease. *J. Lab Clin. Med.* **135,** 122-128.

Kummalue,T. and Friedman,A.D. (2003). Cross-talk between regulators of myeloid development: C/EBPalpha binds and activates the promoter of the PU.1 gene. *J. Leukoc. Biol.* **74,** 464-470.

Kuribayashi,F., Nunoi,H., Wakamatsu,K., Tsunawaki,S., Sato,K., Ito,T., and Sumimoto,H. (2002). The adaptor protein p40(phox) as a positive regulator of the superoxide-producing phagocyte oxidase. *EMBO J.* **21,** 6312-6320.

Kwong,C.H., Malech,H.L., Rotrosen,D., and Leto,T.L. (1993). Regulation of the human neutrophil NADPH oxidase by rho-related G-proteins. *Biochemistry* **32,** 5711-5717.

Lange,C. and Blankenstein,T. (1997). Loss of retroviral gene expression in bone marrow reconstituted mice correlates with down-regulation of gene expression in long-term culture initiating cells. *Gene Ther.* **4,** 303-308.

Leto,T.L., Adams,A.G., and de,M., I (1994). Assembly of the phagocyte NADPH oxidase: binding of Src homology 3 domains to proline-rich targets. *Proc. Natl. Acad. Sci. U. S. A* **91,** 10650-10654.

Leusen,J.H., Verhoeven,A.J., and Roos,D. (1996). Interactions between the components of the human NADPH oxidase: a review about the intrigues in the phox family. *Front Biosci.* **1,** d72-d90.

Levinson,B., Khoury,G., Woude,G.V., and Gruss,P. (1982). Activation of SV40 genome by 72-base pair tandem repeats of Moloney sarcoma virus. *Nature* **295,** 568-572.

Lewis,P.F. and Emerman,M. (1994). Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J. Virol.* **68,** 510-516.

Li,F., Linton,G.F., Sekhsaria,S., Whiting-Theobald,N., Katkin,J.P., Gallin,J.I., and Malech,H.L. (1994). CD34+ peripheral blood progenitors as a target for genetic correction of the two flavocytochrome b558 defective forms of chronic granulomatous disease. *Blood* **84,** 53-58.

Li,S.L., Schlegel,W., Valente,A.J., and Clark,R.A. (1999). Critical flanking sequences of PU.1 binding sites in myeloid-specific promoters. *J. Biol. Chem.* **274,** 32453-32460.

Li,S.L., Valente,A.J., Zhao,S.J., and Clark,R.A. (1997). PU.1 is essential for p47(phox) promoter activity in myeloid cells. *J. Biol. Chem.* **272,** 17802-17809.

Link,D.C., Gutkind,S.J., Robbins,K.C., and Ley,T.J. (1992). Characterization of the 5' untranslated region of the human c-fgr gene and identification of the major myelomonocytic c-fgr promoter. *Oncogene* **7,** 877-884.

Lopes,L.R., Dagher,M.C., Gutierrez,A., Young,B., Bouin,A.P., Fuchs,A., and Babior,B.M. (2004). Phosphorylated p40PHOX as a negative regulator of NADPH oxidase. *Biochemistry* **43,** 3723-3730.

References

Lopes,L.R., Hoyal,C.R., Knaus,U.G., and Babior,B.M. (1999). Activation of the leukocyte NADPH oxidase by protein kinase C in a partially recombinant cell-free system. *J. Biol. Chem.* **274,** 15533-15537.

Lopez-Rodriguez,C., Botella,L., and Corbi,A.L. (1997). CCAAT-enhancer-binding proteins (C/EBP) regulate the tissue specific activity of the CD11c integrin gene promoter through functional interactions with Sp1 proteins. *J. Biol. Chem.* **272,** 29120-29126.

Luthman,H. and Magnusson,G. (1983). High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res.* **11,** 1295-1308.

M'Rabet,L., Coffer,P., Zwartkruis,F., Franke,B., Segal,A.W., Koenderman,L., and Bos,J.L. (1998). Activation of the small GTPase rap1 in human neutrophils. *Blood* **92,** 2133-2140.

MacGregor,R.R. (2001). Clinical protocol. A phase 1 open-label clinical trial of the safety and tolerability of single escalating doses of autologous CD4 T cells transduced with VRX496 in HIV-positive subjects. *Hum. Gene Ther.* **12,** 2028-2029.

MacKenzie,K.L., Bonham,L., and Symonds,G. (1994). An internal deletion enhances the transcriptional activity of a recombinant retrovirus in hematopoietic cells in vivo. *J. Virol.* **68,** 6924-6932.

Malech,H.L. (1999). Progress in gene therapy for chronic granulomatous disease. *J. Infect. Dis.* **179 Suppl 2,** S318-S325.

Malech,H.L., Bauer,T.R., Jr., and Hickstein,D.D. (1997a). Prospects for gene therapy of neutrophil defects. *Semin. Hematol.* **34,** 355-361.

Malech,H.L., Maples,P.B., Whiting,T.N., Linton,G.F., Sekhsaria,S., Vowells,S.J., Li,F., Miller,J.A., DeCarlo,E., Holland,S.M. et al. (1997b). Prolonged production of NADPH oxidase-corrected granulocytes after gene therapy of chronic granulomatous disease. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 12133-12138.

Malik,P., Krall,W.J., Yu,X.J., Zhou,C., and Kohn,D.B. (1995). Retroviral-mediated gene expression in human myelomonocytic cells: a comparison of hematopoietic cell promoters to viral promoters. *Blood* **86,** 2993-3005.

Maly,F.E., Nakamura,M., Gauchat,J.F., Urwyler,A., Walker,C., Dahinden,C.A., Cross,A.R., Jones,O.T., and de Weck,A.L. (1989). Superoxide-dependent nitroblue tetrazolium reduction and expression of cytochrome b-245 components by human tonsillar B lymphocytes and B cell lines. *J. Immunol.* **142,** 1260-1267.

Mann,R., Mulligan,R.C., and Baltimore,D. (1983). Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33,** 153-159.

Manno,C.S., Pierce,G.F., Arruda,V.R., Glader,B., Ragni,M., Rasko,J.J., Ozelo,M.C., Hoots,K., Blatt,P., Konkle,B. et al. (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat. Med.* **12,** 342-347.

Marden,C., Cunninghame,G.D., Thrasher,A., and Casimir,C. (2003a). A functional ISRE is required for myeloid transcription of the p47(phox) gene. *Biochim. Biophys. Acta* **1630,** 117-122.

Marden,C.M., Stefanidis,D., Cunninghame-Graham,D.S., and Casimir,C.M. (2003b). Differentiation-dependent up-regulation of p47(phox) gene transcription is associated with changes in PU.1 phosphorylation and increased binding affinity. *Biochem. Biophys. Res. Commun.* **305,** 193-202.

Mardiney,M., III, Jackson,S.H., Spratt,S.K., Li,F., Holland,S.M., and Malech,H.L. (1997). Enhanced host defense after gene transfer in the murine p47phox- deficient model of chronic granulomatous disease. *Blood* **89,** 2268-2275.

Maridonneau-Parini,I., Tringale,S.M., and Tauber,A.I. (1986). Identification of distinct activation pathways of the human neutrophil NADPH-oxidase. *J. Immunol.* **137,** 2925-2929.

Marin,M., Noel,D., and Piechaczyk,M. (1997). Towards efficient cell targeting by recombinant retroviruses. *Mol. Med. Today* **3,** 396-403.

Markowitz,D., Goff,S., and Bank,A. (1988). Construction and use of a safe and efficient

amphotropic packaging cell line. *Virology* **167,** 400-406.

Martin,P. and Papayannopoulou,T. (1982). HEL cells: a new human erythroleukemia cell line with spontaneous and induced globin expression. *Science* **216,** 1233-1235.

Mata,M., Chattopadhyay,M., and Fink,D.J. (2006). Gene therapy for the treatment of sensory neuropathy. *Expert. Opin. Biol. Ther.* **6,** 499-507.

Mavria,G., Harrington,K.J., Marshall,C.J., and Porter,C.D. (2005). In vivo efficacy of HSV-TK transcriptionally targeted to the tumour vasculature is augmented by combination with cytotoxic chemotherapy. *J. Gene Med.* **7,** 263-275.

Mavria,G., Jager,U., and Porter,C.D. (2000). Generation of a high titre retroviral vector for endothelial cell-specific gene expression in vivo. *Gene Ther.* **7,** 368-376.

Meischl,C. and Roos,D. (1998). The molecular basis of chronic granulomatous disease. *Springer Semin. Immunopathol.* **19,** 417-434.

Messina,C.G., Reeves,E.P., Roes,J., and Segal,A.W. (2002). Catalase negative Staphylococcus aureus retain virulence in mouse model of chronic granulomatous disease. *FEBS Lett.* **518,** 107-110.

Miksicek,R., Heber,A., Schmid,W., Danesch,U., Posseckert,G., Beato,M., and Schutz,G. (1986). Glucocorticoid responsiveness of the transcriptional enhancer of Moloney murine sarcoma virus. *Cell* **46,** 283-290.

Miller,A.D. (1992). Retroviral vectors. *Curr. Top. Microbiol. Immunol.* **158,** 1-24.

Miller,A.D. and Buttimore,C. (1986). Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell Biol.* **6,** 2895-2902.

Miller,A.D., GarciaJ.V., von,S.N., Lynch,C.M., Wilson,C., and Eiden,M.V. (1991). Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J. Virol.* **65,** 2220-2224.

Miller,N. and Vile,R. (1995). Targeted vectors for gene therapy. *FASEB J.* **9,** 190-199.

Miller,N. and Whelan,J. (1997). Progress in transcriptionally targeted and regulatable vectors for genetic therapy. *Hum. Gene Ther.* **8,** 803-815.

Mitchison,N.A., Harbord,M., Hankin,A., and Roes,J. (2005). Conditional haploinsufficiency of NCF1 (encoding p47(phox)), a signaling gene with a heterozygous phenotype potentially subject to natural selection. *Immunol. Lett.* **97,** 63-67.

Miyazaki,Y., Katamine,S., Kohno,T., Moriuchi,R., Miyamoto,T., and Tomonaga,M. (1993). fgr proto-oncogene is expressed during terminal granulocytic differentiation of human promyelocytic HL60 cells. *Exp. Hematol.* **21,** 366-371.

Mizushima,S. and Nagata,S. (1990). pEF-BOS, a powerful mammalian expression vector. *Nucleic. Acids. Res.* **18,** 5322.

Mizutani,S., Boettiger,D., and Temin,H.M. (1970). A DNA-depenent DNA polymerase and a DNA endonuclease in virions of Rous sarcoma virus. *Nature* **228,** 424-427.

Moelling,K., Bolognesi,D.P., and Bauer,H. (1971). Polypeptides of avian RNA tumor viruses. 3. Purification and identification of a DNA synthesizing enzyme. *Virology* **45,** 298-302.

Morgenstern,J.P. and Land,H. (1990). A series of mammalian expression vectors and characterisation of their expression of a reporter gene in stably and transiently transfected cells. *Nucleic. Acids. Res.* **18,** 1068.

Moulton,K.S., Semple,K., Wu,H., and Glass,C.K. (1994). Cell-specific expression of the macrophage scavenger receptor gene is dependent on PU.1 and a composite AP-1/ets motif. *Mol. Cell Biol.* **14,** 4408-4418.

Mulder,A., Alexander,S., Engelfriet,C.P., von,d.B., and Strominger,J.L. (1981). Characterization, by immunoprecipitation, of myeloid- and monocyte-specific antigens present on the human promyelocytic cell line (HL-60) in three stages of differentiation. *Proc. Natl. Acad. Sci. U. S. A.* **78,** 5091-5095.

Nagamura-Inoue,T., Tamura,T., and Ozato,K. (2001). Transcription factors that regulate growth and differentiation of myeloid cells. *Int. Rev. Immunol.* **20,** 83-105.

References

Nakamura,R., Sumimoto,H., Mizuki,K., Hata,K., Ago,T., Kitajima,S., Takeshige,K., Sakaki,Y., and Ito,T. (1998). The PC motif: a novel and evolutionarily conserved sequence involved in interaction between p40phox and p67phox, SH3 domain-containing cytosolic factors of the phagocyte NADPH oxidase. *Eur. J. Biochem.* **251,** 583-589.

Nakanishi,A., Imajoh-Ohmi,S., Fujinawa,T., Kikuchi,H., and Kanegasaki,S. (1992). Direct evidence for interaction between COOH-terminal regions of cytochrome b558 subunits and cytosolic 47-kDa protein during activation of an O(2-)-generating system in neutrophils. *J. Biol. Chem.* **267,** 19072-19074.

Naldini,L., Blomer,U., Gallay,P., Ory,D., Mulligan,R., Gage,F.H., Verma,I.M., and Trono,D. (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263-267.

Nanda,A., Curnutte,J.T., and Grinstein,S. (1994). Activation of H+ conductance in neutrophils requires assembly of components of the respiratory burst oxidase but not its redox function. *J. Clin. Invest* **93,** 1770-1775.

Nanda,A., Grinstein,S., and Curnutte,J.T. (1993). Abnormal activation of H+ conductance in NADPH oxidase-defective neutrophils. *Proc. Natl. Acad. Sci. U. S. A* **90,** 760-764.

Natsuka,S., Akira,S., Nishio,Y., Hashimoto,S., Sugita,T., Isshiki,H., and Kishimoto,T. (1992). Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. *Blood* **79,** 460-466.

Nauseef,W.M. (1999). The NADPH-dependent oxidase of phagocytes. *Proc. Assoc. Am. Physicians* **111,** 373-382.

Nauseef,W.M. (2004). Assembly of the phagocyte NADPH oxidase. *Histochem. Cell Biol.* **122,** 277-291.

Nauseef,W.M., McCormick,S., Renee,J., Leidal,K.G., and Clark,R.A. (1993). Functional domain in an arginine-rich carboxyl-terminal region of p47phox. *J. Biol. Chem.* **268,** 23646-23651.

Naviaux,R.K., Costanzi,E., Haas,M., and Verma,I.M. (1996). The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J. Virol.* **70,** 5701-5705.

Nerlov,C. and Ziff,E.B. (1995). CCAAT/enhancer binding protein-alpha amino acid motifs with dual TBP and TFIIB binding ability co-operate to activate transcription in both yeast and mammalian cells. *EMBO J.* **14,** 4318-4328.

Newburger,P.E., Ezekowitz,R.A., Whitney,C., Wright,J., and Orkin,S.H. (1988). Induction of phagocyte cytochrome b heavy chain gene expression by interferon gamma. *Proc. Natl. Acad. Sci. U. S. A* **85,** 5215-5219.

Nielsen,A.L., Pallisgaard,N., Pedersen,F.S., and Jorgensen,P. (1992). Murine helix-loop-helix transcriptional activator proteins binding to the E-box motif of the Akv murine leukemia virus enhancer identified by cDNA cloning. *Mol. Cell Biol.* **12,** 3449-3459.

Noack,D., Rae,J., Cross,A.R., Munoz,J., Salmen,S., Mendoza,J.A., Rossi,N., Curnutte,J.T., and Heyworth,P.G. (1999). Autosomal recessive chronic granulomatous disease caused by novel mutations in NCF-2, the gene encoding the p67-phox component of phagocyte NADPH oxidase. *Hum. Genet.* **105,** 460-467.

Noti,J.D., Johnson,A.K., and Dillon,J.D. (2000). Structural and functional characterization of the leukocyte integrin gene CD11d. Essential role of Sp1 and Sp3. *J. Biol. Chem.* **275,** 8959-8969.

Noti,J.D., Reinemann,B.C., and Petrus,M.N. (1996). Sp1 binds two sites in the CD11c promoter in vivo specifically in myeloid cells and cooperates with AP1 to activate transcription. *Mol. Cell Biol.* **16,** 2940-2950.

Nuchprayoon,I., Meyers,S., Scott,L.M., Suzow,J., Hiebert, and Friedman,A.D. (1994). PEBP2/CBF, the murine homolog of the human myeloid AML1 and PEBP2 beta/CBF beta proto-oncoproteins, regulates the murine myeloperoxidase and neutrophil elastase genes in immature myeloid cells. *Mol. Cell Biol.* **14,** 5558-5568.

References

Nuchprayoon,I., Simkevich,C.P., Luo,M., Friedman,A.D., and Rosmarin,A.G. (1997). GABP cooperates with c-Myb and C/EBP to activate the neutrophil elastase promoter. *Blood* **89,** 4546-4554.

Nunoi,H., Rotrosen,D., Gallin,J.I., and Malech,H.L. (1988). Two forms of autosomal chronic granulomatous disease lack distinct neutrophil cytosol factors. *Science* **242,** 1298-1301.

Ochs,H.D., Smith,E., and Puck,J. (1999). *Primary immunodeficiency diseases : a molecular and genetic approach.* New York ; Oxford : Oxford University Press.

Oelgeschlager,M., Nuchprayoon,I., Luscher,B., and Friedman (1996). C/EBP, c-Myb, and PU.1 cooperate to regulate the neutrophil elastase promoter. *Mol. Cell Biol.* **16,** 4717-4725.

Ogawa,E., Inuzuka,M., Maruyama,M., Satake,M., Naito-Fujimoto,M., Ito,Y., and Shigesada,K. (1993a). Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2 alpha. *Virology* **194,** 314-331.

Ogawa,E., Maruyama,M., Kagoshima,H., Inuzuka,M., Lu,J., Satake,M., Shigesada,K., and Ito,Y. (1993b). PEBP2/PEA2 represents a family of transcription factors homologous to the products of the Drosophila runt gene and the human AML1 gene. *Proc. Natl. Acad. Sci. U. S. A* **90,** 6859-6863.

Okamura,N., Curnutte,J.T., Roberts,R.L., and Babior,B.M. (1988). Relationship of protein phosphorylation to the activation of the respiratory burst in human neutrophils. Defects in the phosphorylation of a group of closely related 48-kDa proteins in two forms of chronic granulomatous disease. *J. Biol. Chem.* **263,** 6777-6782.

Ott,M.G., Schmidt,M., Schwarzwaelder,K., Stein,S., Siler,U., Koehl,U., Glimm,H., Kuhlcke,K., Schilz,A., Kunkel,H. et al. (2006). Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat. Med.* **12,** 401-409.

Ott,M.G., Seger,R., Stein,S., Siler,U., Hoelzer,D., and Grez,M. (2007). Advances in the treatment of Chronic Granulomatous Disease by gene therapy. *Curr. Gene Ther.* **7,** 155-161.

Pahl,H.L., Burn,T.C., and Tenen,D.G. (1991). Optimization of transient transfection into human myeloid cell lines using a luciferase reporter gene. *Exp. Hematol.* **19,** 1038-1041.

Pahl,H.L., Rosmarin,A.G., and Tenen,D.G. (1992). Characterization of the myeloid-specific CD11b promoter. *Blood* **79,** 865-870.

Painter,R.G., Lanson,N.A., Jr., Jin,Z., Park,F., and Wang,G. (2005). Conditional expression of a suicide gene by the telomere reverse transcriptase promoter for potential post-therapeutic deletion of tumorigenesis. *Cancer Sci.* **96,** 607-613.

Palu,G., Parolin,C., Takeuchi,Y., and Pizzato,M. (2000). Progress with retroviral gene vectors. *Rev. Med. Virol.* **10,** 185-202.

Pannell,D. and Ellis,J. (2001). Silencing of gene expression: implications for design of retrovirus vectors. *Rev. Med. Virol.* **11,** 205-217.

Park,J.W. and Babior,B.M. (1997). Activation of the leukocyte NADPH oxidase subunit p47phox by protein kinase C. A phosphorylation-dependent change in the conformation of the C-terminal end of p47phox. *Biochemistry* **36,** 7474-7480.

Park,J.W., Benna,J.E., Scott,K.E., Christensen,B.L., Chanock,S.J., and Babior,B.M. (1994). Isolation of a complex of respiratory burst oxidase components from resting neutrophil cytosol. *Biochemistry* **33,** 2907-2911.

Park,J.W., Hoyal,C.R., Benna,J.E., and Babior,B.M. (1997). Kinase-dependent activation of the leukocyte NADPH oxidase in a cell-free system. Phosphorylation of membranes and p47(PHOX) during oxidase activation. *J. Biol. Chem.* **272,** 11035-11043.

Park,J.W., Ma,M., Ruedi,J.M., Smith,R.M., and Babior,B.M. (1992). The cytosolic components of the respiratory burst oxidase exist as a M(r) approximately 240,000 complex that acquires a membrane-binding site during

activation of the oxidase in a cell-free system. *J. Biol. Chem.* **267,** 17327-17332.

Parkos,C.A., Allen,R.A., Cochrane,C.G., and Jesaitis,A.J. (1987). Purified cytochrome b from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000. *J. Clin. Invest* **80,** 732-742.

Parkos,C.A., Dinauer,M.C., Jesaitis,A.J., Orkin,S.H., and Curnutte,J.T. (1989). Absence of both the 91kD and 22kD subunits of human neutrophil cytochrome b in two genetic forms of chronic granulomatous disease. *Blood* **73,** 1416-1420.

Patino,P.J., Perez,J.E., Lopez,J.A., Condino-Neto,A., Grumach,A.S., Botero,J.H., Curnutte,J.T., and Garcia,d.O. (1999). Molecular analysis of chronic granulomatous disease caused by defects in gp91-phox. *Hum. Mutat.* **13,** 29-37.

Pear,W.S., Nolan,G.P., Scott,M.L., and Baltimore,D. (1993). Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8392-8396.

Perez,C., Wietzerbin,J., and Benech,P.D. (1993). Two cis-DNA elements involved in myeloid-cell-specific expression and gamma interferon (IFN-gamma) activation of the human high-affinity Fc gamma receptor gene: a novel IFN regulatory mechanism. *Mol. Cell Biol.* **13,** 2182-2192.

Persons,D.A. and Nienhuis,A.W. (2000). Gene therapy for the hemoglobin disorders: past, present, and future. *Proc. Natl. Acad. Sci. U. S. A* **97,** 5022-5024.

Philpott,N.J. and Thrasher,A.J. (2007). Use of nonintegrating lentiviral vectors for gene therapy. *Hum. Gene Ther.* **18,** 483-489.

Pick,E., Gorzalczany,Y., and Engel,S. (1993). Role of the rac1 p21-GDP-dissociation inhibitor for rho heterodimer in the activation of the superoxide-forming NADPH oxidase of macrophages. *Eur. J. Biochem.* **217,** 441-455.

Pollock,J.D., Williams,D.A., Gifford,M.A., Li,L.L., Du,X., Fisherman,J., Orkin,S.H., Doerschuk,C.M., and Dinauer,M.C. (1995). Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat. Genet.* **9,** 202-209.

Porter,C.D., Parkar,M.H., Collins,M.K., Levinsky,R.J., and Kinnon,C. (1996a). Efficient retroviral transduction of human bone marrow progenitor and long-term culture-initiating cells: partial reconstitution of cells from patients with X-linked chronic granulomatous disease by gp91-phox expression. *Blood* **87,** 3722-3730.

Porter,C.D., Parkar,M.H., and Kinnon,C. (1996b). Identification of a donor splice site mutation leading to loss of p22- phox exon 5 in autosomal chronic granulomatous disease. *Hum. Mutat.* **7,** 374.

Porter,C.D., Parkar,M.H., Levinsky,R.J., Collins,M.K., and Kinnon,C. (1993). X-linked chronic granulomatous disease: correction of NADPH oxidase defect by retrovirus-mediated expression of gp91-phox. *Blood* **82,** 2196-2202.

Prigmore,E., Ahmed,S., Best,A., Kozma,R., Manser,E., Segal,A.W., and Lim,L. (1995). A 68-kDa kinase and NADPH oxidase component p67phox are targets for Cdc42Hs and Rac1 in neutrophils. *J. Biol. Chem.* **270,** 10717-10722.

Proudfoot,N.J. (1986). Transcriptional interference and termination between duplicated alpha-globin gene constructs suggests a novel mechanism for gene regulation. *Nature* **322,** 562-565.

Qasim,W., Gaspar,H.B., and Thrasher,A.J. (2004). Gene therapy for severe combined immune deficiency. *Expert. Rev. Mol. Med.* **2004,** 1-15.

Qu,A.Y., Nanda,A., Curnutte,J.T., and Grinstein,S. (1994). Development of a H(+)-selective conductance during granulocytic differentiation of HL-60 cells. *Am. J. Physiol* **266,** C1263-C1270.

Quinn,M.T., Parkos,C.A., Walker,L., Orkin,S.H., Dinauer,M.C., and Jesaitis,A.J. (1989). Association of a Ras-related protein with cytochrome b of human neutrophils. *Nature* **342,** 198-200.

Radler,J.O., Koltover,I., Salditt,T., and Safinya,C.R. (1997). Structure of DNA-cationic liposome complexes: DNA intercalation in

multilamellar membranes in distinct interhelical packing regimes. *Science* **275,** 810-814.

Rae,J., Newburger,P.E., Dinauer,M.C., Noack,D., Hopkins,P.J., Kuruto,R., and Curnutte,J.T. (1998). X-Linked chronic granulomatous disease: mutations in the CYBB gene encoding the gp91-phox component of respiratory-burst oxidase. *Am. J. Hum. Genet.* **62,** 1320-1331.

Rae,J., Noack,D., Heyworth,P.G., Ellis,B.A., Curnutte,J.T., and Cross,A.R. (2000). Molecular analysis of 9 new families with chronic granulomatous disease caused by mutations in CYBA, the gene encoding p22(phox). *Blood* **96,** 1106-1112.

Raper,S.E., Chirmule,N., Lee,F.S., Wivel,N.A., Bagg,A., Gao,G.P., Wilson,J.M., and Batshaw,M.L. (2003). Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol. Genet. Metab* **80,** 148-158.

Reddy,M.A., Yang,B.S., Yue,X., Barnett,C.J., Ross,I.L., Sweet,M.J., Hume,D.A., and Ostrowski,M.C. (1994). Opposing actions of c-ets/PU.1 and c-myb protooncogene products in regulating the macrophage-specific promoters of the human and mouse colony-stimulating factor-1 receptor (c-fms) genes. *J. Exp. Med.* **180,** 2309-2319.

Reisman,D. (1990). Nuclear factor-1 (NF-1) binds to multiple sites within the transcriptional enhancer of Moloney murine leukemia virus. *FEBS Lett.* **277,** 209-211.

Richardson,M.P., Ayliffe,M.J., Helbert,M., and Davies,E.G. (1998). A simple flow cytometry assay using dihydrorhodamine for the measurement of the neutrophil respiratory burst in whole blood: comparison with the quantitative nitrobluetetrazolium test. *J Immunol Methods* **219,** 187-193.

Ridley,A.J., Paterson,H.F., Johnston,C.L., Diekmann,D., and Hall,A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70,** 401-410.

Robbins,P.B., Skelton,D.C., Yu,X.J., Halene,S., Leonard,E.H., and Kohn,D.B. (1998). Consistent, persistent expression from modified retroviral vectors in murine hematopoietic stem cells. *Proc. Natl. Acad. Sci. U. S. A* **95,** 10182-10187.

Robbins,P.B., Yu,X.J., Skelton,D.M., Pepper,K.A., Wasserman,R.M., Zhu,L., and Kohn,D.B. (1997). Increased probability of expression from modified retroviral vectors in embryonal stem cells and embryonal carcinoma cells. *J. Virol.* **71,** 9466-9474.

Rodaway,A.R., Teahan,C.G., Casimir,C.M., Segal,A.W., and Bentley (1990). Characterization of the 47-kilodalton autosomal chronic granulomatous disease protein: tissue-specific expression and transcriptional control by retinoic acid. *Mol. Cell Biol.* **10,** 5388-5396.

Roe,T., Reynolds,T.C., Yu,G., and Brown,P.O. (1993). Integration of murine leukemia virus DNA depends on mitosis. *EMBO J.* **12,** 2099-2108.

Roesler,J., Brenner,S., Bukovsky,A.A., Whiting-Theobald,N., Dull,T., Kelly,M., Civin,C.I., and Malech,H.L. (2002). Third-generation, self-inactivating gp91(phox) lentivector corrects the oxidase defect in NOD/SCID mouse-repopulating peripheral blood-mobilized CD34+ cells from patients with X-linked chronic granulomatous disease. *Blood* **100,** 4381-4390.

Roesler,J., Curnutte,J.T., Rae,J., Barrett,D., Patino,P., Chanock,S.J., and Goerlach,A. (2000). Recombination events between the p47-phox gene and its highly homologous pseudogenes are the main cause of autosomal recessive chronic granulomatous disease. *Blood* **95,** 2150-2156.

Roesler,J., Heyden,S., Burdelski,M., Schafer,H., Kreth,H.W., Lehmann,R., Paul,D., Marzahn,J., Gahr,M., and Rosen-Wolff,A. (1999). Uncommon missense and splice mutations and resulting biochemical phenotypes in German patients with X-linked chronic granulomatous disease. *Exp. Hematol.* **27,** 505-511.

Roos,D. (1994). The genetic basis of chronic granulomatous disease. *Immunol. Rev.* **138,** 121-157.

Roos,D., de Boer,M., Kuribayashi,F., Meischl,C., Weening,R.S., Segal,A.W.,

References

Ahlin,A., Nemet,K., Hossle,J.P., Bernatowska-Matuszkiewicz,E. et al. (1996). Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. *Blood* **87,** 1663-1681.

Rosen,G.M., Pou,S., Ramos,C.L., Cohen,M.S., and Britigan,B.E. (1995). Free radicals and phagocytic cells. *FASEB J.* **9,** 200-209.

Rosmarin,A.G., Caprio,D., Levy,R., and Simkevich,C. (1995). CD18 (beta 2 leukocyte integrin) promoter requires PU.1 transcription factor for myeloid activity. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 801-805.

Rosmarin,A.G., Yang,Z., and Resendes,K.K. (2005). Transcriptional regulation in myelopoiesis: Hematopoietic fate choice, myeloid differentiation, and leukemogenesis. *Exp. Hematol.* **33,** 131-143.

Rotrosen,D. and Leto,T.L. (1990). Phosphorylation of neutrophil 47-kDa cytosolic oxidase factor. Translocation to membrane is associated with distinct phosphorylation events. *J. Biol. Chem.* **265,** 19910-19915.

Rotrosen,D., Yeung,C.L., and Katkin,J.P. (1993). Production of recombinant cytochrome b558 allows reconstitution of the phagocyte NADPH oxidase solely from recombinant proteins. *J. Biol. Chem.* **268,** 14256-14260.

Sadat,M.A., Pech,N., Saulnier,S., Leroy,B.A., Hossle,J.P., Grez,M., and Dinauer,M.C. (2003). Long-term high-level reconstitution of NADPH oxidase activity in murine X-linked chronic granulomatous disease using a bicistronic vector expressing gp91phox and a Delta LNGFR cell surface marker. *Hum. Gene Ther.* **14,** 651-666.

Sadelain,M., Frassoni,F., and Riviere,I. (2000). Issues in the manufacture and transplantation of genetically modified hematopoietic stem cells. *Curr. Opin. Hematol.* **7,** 364-377.

Sadelain,M., Wang,C.H., Antoniou,M., Grosveld,F., and Mulligan,R.C. (1995). Generation of a high-titer retroviral vector capable of expressing high levels of the human beta-globin gene. *Proc. Natl. Acad. Sci. U. S. A* **92,** 6728-6732.

Sambucetti,L.C., Cherrington,J.M., Wilkinson,G.W., and Mocarski,E.S. (1989). NF-kappa B activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. *EMBO J.* **8,** 4251-4258.

Sathyamoorthy,M., de,M., I, Adams,A.G., and Leto,T.L. (1997). p40(phox) down-regulates NADPH oxidase activity through interactions with its SH3 domain. *J. Biol. Chem.* **272,** 9141-9146.

Schambach,A., Mueller,D., Galla,M., Verstegen,M.M., Wagemaker,G., Loew,R., Baum,C., and Bohne,J. (2006). Overcoming promoter competition in packaging cells improves production of self-inactivating retroviral vectors. *Gene Ther.* **13,** 1524-1533.

Scherer,W.F., Syverton,J.T., and Gey,G.O. (1953). Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. *J. Exp. Med.* **97,** 695-710.

Schilz,A.J., Brouns,G., Knoss,H., Ottmann,O.G., Hoelzer,D., Fauser,A.A., Thrasher,A.J., and Grez,M. (1998). High efficiency gene transfer to human hematopoietic SCID-repopulating cells under serum-free conditions. *Blood* **92,** 3163-3171.

Schneider,U., Schwenk,H.U., and Bornkamm,G. (1977). Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. *Int. J. Cancer* **19,** 621-626.

Schroder,A.R., Shinn,P., Chen,H., Berry,C., Ecker,J.R., and Bushman,F. (2002). HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* **110,** 521-529.

Schwickerath,O., Brouns,G., Thrasher,A., Kinnon,C., Roes,J., and Casimir,C. (2004). Enhancer-deleted retroviral vectors restore high levels of superoxide generation in a mouse model of CGD. *J. Gene Med.* **6,** 603-615.

Scott,L.M., Civin,C.I., Rorth,P., and Friedman,A.D. (1992). A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. *Blood* **80,** 1725-1735.

References

Segal,A.W. (1987). Absence of both cytochrome b-245 subunits from neutrophils in X-linked chronic granulomatous disease. *Nature* **326,** 88-91.

Segal,A.W. (2005). How neutrophils kill microbes. *Annu. Rev. Immunol.* **23,** 197-223.

Segal,A.W. and Abo,A. (1993). The biochemical basis of the NADPH oxidase of phagocytes. *Trends Biochem. Sci.* **18,** 43-47.

Segal,A.W., Geisow,M., Garcia,R., Harper,A., and Miller,R. (1981). The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH. *Nature* **290,** 406-409.

Segal,A.W., Heyworth,P.G., Cockcroft,S., and Barrowman,M.M. (1985). Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylate a Mr-44,000 protein. *Nature* **316,** 547-549.

Segal,B.H., Doherty,T.M., Wynn,T.A., Cheever,A.W., Sher,A., and Holland,S.M. (1999). The p47(phox-/-) mouse model of chronic granulomatous disease has normal granuloma formation and cytokine responses to Mycobacterium avium and Schistosoma mansoni eggs. *Infect. Immun.* **67,** 1659-1665.

Segal,B.H., Leto,T.L., Gallin,J.I., Malech,H.L., and Holland,S.M. (2000). Genetic, biochemical, and clinical features of chronic granulomatous disease. *Medicine (Baltimore)* **79,** 170-200.

Seger,R.A. and Ezekowitz,R.A. (1994). Treatment of chronic granulomatous disease. *Immunodeficiency* **5,** 113-130.

Sekhsaria,S., Fleisher,T.A., Vowells,S., Brown,M., Miller,J., Gordon,I., Blaese,R.M., Dunbar,C.E., Leitman,S., and Malech,H.L. (1996). Granulocyte colony-stimulating factor recruitment of CD34+ progenitors to peripheral blood: impaired mobilization in chronic granulomatous disease and adenosine deaminase--deficient severe combined immunodeficiency disease patients. *Blood* **88,** 1104-1112.

Sekhsaria,S. and Malech,H.L. (1993). Recombinant human stem cell factor enhances myeloid colony growth from human peripheral blood progenitors. *Blood* **81,** 2125-2130.

Shapiro,L.H., Ashmun,R.A., Roberts,W.M., and Look,A.T. (1991). Separate promoters control transcription of the human aminopeptidase N gene in myeloid and intestinal epithelial cells. *J. Biol. Chem.* **266,** 11999-12007.

Shelley,C.S. and Arnaout,M.A. (1991). The promoter of the CD11b gene directs myeloid-specific and developmentally regulated expression. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 10525-10529.

Shivdasani,R.A. and Orkin,S.H. (1996). The transcriptional control of hematopoiesis. *Blood* **87,** 4025-4039.

Simon,M.C. (1998). PU.1 and hematopoiesis: lessons learned from gene targeting experiments. *Semin. Immunol.* **10,** 111-118.

Simon,M.C., Olson,M., Scott,E., Hack,A., Su,G., and Singh,H. (1996). Terminal myeloid gene expression and differentiation requires the transcription factor PU.1. *Curr. Top. Microbiol. Immunol.* **211,** 113-119.

Sinn,P.L., Sauter,S.L., and McCray,P.B., Jr. (2005). Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors--design, biosafety, and production. *Gene Ther.* **12,** 1089-1098.

Skalnik,D.G. (2002). Transcriptional mechanisms regulating myeloid-specific genes. *Gene* **284,** 1-21.

Smith,L.T., Hohaus,S., Gonzalez,D.A., Dziennis,S.E., and Tenen,D.G. (1996a). PU.1 (Spi-1) and C/EBP alpha regulate the granulocyte colony- stimulating factor receptor promoter in myeloid cells. *Blood* **88,** 1234-1247.

Smith,R.M., Connor,J.A., Chen,L.M., and Babior,B.M. (1996b). The cytosolic subunit p67phox contains an NADPH-binding site that participates in catalysis by the leukocyte NADPH oxidase. *J. Clin. Invest* **98,** 977-983.

Someya,A., Nagaoka,I., Nunoi,H., and Yamashita,T. (1996). Translocation of guinea pig p40-phox during activation of NADPH oxidase. *Biochim. Biophys. Acta* **1277,** 217-225.

Soneoka,Y., Cannon,P.M., Ramsdale,E.E., Griffiths,J.C., Romano,G., Kingsman,S.M., and Kingsman,A.J. (1995). A transient three-

plasmid expression system for the production of high titer retroviral vectors. *Nucleic. Acids. Res.* **23,** 628-633.

Soriano,P., Friedrich,G., and Lawinger,P. (1991). Promoter interactions in retrovirus vectors introduced into fibroblasts and embryonic stem cells. *J. Virol.* **65,** 2314-2319.

Srikanth,S. and Rado,T.A. (1994). A 30-base pair element is responsible for the myeloid-specific activity of the human neutrophil elastase promoter. *J. Biol. Chem.* **269,** 32626-32633.

Srikanth,S. and Rado,T.A. (1998). PU.1 regulates the expression of the human neutrophil elastase gene. *Biochim. Biophys. Acta* **1398,** 215-223.

Stein,S., Siler,U., Ott,M.G., Seger,R., and Grez,M. (2006). Gene therapy for chronic granulomatous disease. *Curr. Opin. Mol. Ther.* **8,** 415-422.

Stuhlmann,H., Cone,R., Mulligan,R.C., and Jaenisch,R. (1984). Introduction of a selectable gene into different animal tissue by a retrovirus recombinant vector. *Proc. Natl. Acad. Sci. U. S. A* **81,** 7151-7155.

Sturrock,A., Franklin,K.F., and Hoidal,J.R. (1996). Human proteinase-3 expression is regulated by PU.1 in conjunction with a cytidine-rich element. *J. Biol. Chem.* **271,** 32392-32402.

Sturrock,A., Franklin,K.F., Norman,K., and Hoidal,J.R. (2004). Human leukocyte elastase gene expression is regulated by PU.1 in conjunction with closely associated cytidine-rich and Myb binding sites. *Biochim. Biophys. Acta* **1676,** 104-111.

Sumimoto,H., Hata,K., Mizuki,K., Ito,T., Kage,Y., Sakaki,Y., Fukumaki,Y., Nakamura,M., and Takeshige,K. (1996). Assembly and activation of the phagocyte NADPH oxidase. Specific interaction of the N-terminal Src homology 3 domain of p47phox with p22phox is required for activation of the NADPH oxidase. *J. Biol. Chem.* **271,** 22152-22158.

Sumimoto,H., Ueno,N., Yamasaki,T., Taura,M., and Takeya,R. (2004). Molecular mechanism underlying activation of superoxide-producing NADPH oxidases: roles for their regulatory proteins. *Jpn. J. Infect. Dis.* **57,** S24-S25.

Sun,W., Graves,B.J., and Speck,N.A. (1995). Transactivation of the Moloney murine leukemia virus and T-cell receptor beta-chain enhancers by cbf and ets requires intact binding sites for both proteins. *J. Virol.* **69,** 4941-4949.

Sundstrom,C. and Nilsson,K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer* **17,** 565-577.

Suzuki,S., Kumatori,A., Haagen,I.A., Fujii,Y., Sadat,M.A., Jun,H.L., Tsuji,Y., Roos,D., and Nakamura,M. (1998). PU.1 as an essential activator for the expression of gp91(phox) gene in human peripheral neutrophils, monocytes, and B lymphocytes. *Proc. Natl. Acad. Sci. U. S. A* **95,** 6085-6090.

Swain,S.D., Helgerson,S.L., Davis,A.R., Nelson,L.K., and Quinn,M.T. (1997). Analysis of activation-induced conformational changes in p47phox using tryptophan fluorescence spectroscopy. *J. Biol. Chem.* **272,** 29502-29510.

Takahashi,H., Nukiwa,T., Basset,P., and Crystal,R.G. (1988a). Myelomonocytic cell lineage expression of the neutrophil elastase gene. *J. Biol. Chem.* **263,** 2543-2547.

Takahashi,H., Nukiwa,T., Yoshimura,K., Quick,C.D., States,D.J., Holmes,M.D., Whang-Peng,J., Knutsen,T., and Crystal,R.G. (1988b). Structure of the human neutrophil elastase gene. *J. Biol. Chem.* **263,** 14739-14747.

Takeuchi,Y. and Pizzato,M. (2000). Retrovirus vectors. *Adv. Exp. Med. Biol.* **465,** 23-35.

Takeya,R. and Sumimoto,H. (2003). Molecular mechanism for activation of superoxide-producing NADPH oxidases. *Mol. Cells* **16,** 271-277.

Tang,H., Kuhen,K.L., and Wong-Staal,F. (1999). Lentivirus replication and regulation. *Annu. Rev. Genet.* **33,** 133-170.

Teahan,C.G., Totty,N., Casimir,C.M., and Segal,A.W. (1990). Purification of the 47 kDa phosphoprotein associated with the NADPH oxidase of human neutrophils. *Biochem. J.* **267,** 485-489.

References

Temin,H.M. and Mizutani,S. (1970). RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature* **226,** 1211-1213.

Tenen,D.G. (2003). Disruption of differentiation in human cancer: AML shows the way. *Nat. Rev. Cancer* **3,** 89-101.

Tenen,D.G., Hromas,R., Licht,J.D., and Zhang,D.E. (1997). Transcription factors, normal myeloid development, and leukemia. *Blood* **90,** 489-519.

Themis,M., Waddington,S.N., Schmidt,M., von,K.C., Wang,Y., Al-Allaf,F., Gregory,L.G., Nivsarkar,M., Themis,M., Holder,M.V. et al. (2005). Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice. *Mol. Ther.* **12,** 763-771.

Thomas,C.E., Ehrhardt,A., and Kay,M.A. (2003). Progress and problems with the use of viral vectors for gene therapy. *Nat. Rev. Genet.* **4,** 346-358.

Thornhill,S.I., Schambach,A., Howe,S.J., Ulaganathan,M., Grassman,E., Williams,D., Schiedlmeier,B., Sebire,N.J., Gaspar,H.B., Kinnon,C. et al. (2008). Self-inactivating gammaretroviral vectors for gene therapy of X-linked severe combined immunodeficiency. *Mol. Ther.* **16,** 590-598.

Thrasher,A., Chetty,M., Casimir,C., and Segal,A.W. (1992). Restoration of superoxide generation to a chronic granulomatous disease-derived B-cell line by retrovirus mediated gene transfer. *Blood* **80,** 1125-1129.

Thrasher,A., Segal,A., and Casimir,C. (1993). Chronic granulomatous disease: towards gene therapy. *Immunodeficiency* **4,** 327-333.

Thrasher,A.J., Casimir,C.M., Kinnon,C., Morgan,G., Segal,A.W., and Levinsky,R.J. (1995). Gene transfer to primary chronic granulomatous disease monocytes. *Lancet* **346,** 92-93.

Thrasher,A.J., Gaspar,H.B., Baum,C., Modlich,U., Schambach,A., Candotti,F., Otsu,M., Sorrentino,B., Scobie,L., Cameron,E. et al. (2006). Gene therapy: X-SCID transgene leukaemogenicity. *Nature* **443,** E5-E6.

Thrasher,A.J., Keep,N.H., Wientjes,F., and Segal,A.W. (1994). Chronic granulomatous disease. *Biochim. Biophys. Acta* **1227,** 1-24.

Tsukiyama,T., Ueda,H., Hirose,S., and Niwa,O. (1992). Embryonal long terminal repeat-binding protein is a murine homolog of FTZ-F1, a member of the steroid receptor superfamily. *Mol. Cell Biol.* **12,** 1286-1291.

Tsunawaki,S., Kagara,S., Yoshikawa,K., Yoshida,L.S., Kuratsuji,T., and Namiki,H. (1996). Involvement of p40phox in activation of phagocyte NADPH oxidase through association of its carboxyl-terminal, but not its amino-terminal, with p67phox. *J. Exp. Med.* **184,** 893-902.

Tsunawaki,S., Mizunari,H., Nagata,M., Tatsuzawa,O., and Kuratsuji,T. (1994). A novel cytosolic component, p40phox, of respiratory burst oxidase associates with p67phox and is absent in patients with chronic granulomatous disease who lack p67phox. *Biochem. Biophys. Res. Commun.* **199,** 1378-1387.

Tsutsumi-Ishii,Y., Hasebe,T., and Nagaoka,I. (2000). Role of CCAAT/enhancer-binding protein site in transcription of human neutrophil peptide-1 and -3 defensin genes. *J. Immunol.* **164,** 3264-3273.

Tucker,K.A., Lilly,M.B., Heck,L., Jr., and Rado,T.A. (1987). Characterization of a new human diploid myeloid leukemia cell line (PLB-985) with granulocytic and monocytic differentiating capacity. *Blood* **70,** 372-378.

Uduehi,A., Mailhos,C., Truman,H., Thrasher,A.J., Kinnon,C., and Hart,S.L. (2003). Enhancement of integrin-mediated transfection of haematopoietic cells with a synthetic vector system. *Biotechnol. Appl. Biochem.* **38,** 201-209.

Vazquez,N., Lehrnbecher,T., Chen,R., Christensen,B.L., Gallin,J.I., Malech,H., Holland,S., Zhu,S., and Chanock,S.J. (2001). Mutational analysis of patients with p47-phox-deficient chronic granulomatous disease: The significance of recombination events between the p47-phox gene (NCF1) and its highly homologous pseudogenes. *Exp. Hematol.* **29,** 234-243.

References

Vergnaud,S., Paclet,M.H., el,B.J., Pocidalo,M.A., and Morel,F. (2000). Complementation of NADPH oxidase in p67-phox-deficient CGD patients p67-phox/p40-phox interaction. *Eur. J. Biochem.* **267,** 1059-1067.

Verma,I.M. and Somia,N. (1997). Gene therapy -- promises, problems and prospects [news]. *Nature* **389,** 239-242.

Vile,R., Miller,N., Chernajovsky,Y., and Hart,I. (1994a). A comparison of the properties of different retroviral vectors containing the murine tyrosinase promoter to achieve transcriptionally targeted expression of the HSVtk or IL-2 genes. *Gene Ther.* **1,** 307-316.

Vile,R.G., Diaz,R.M., Miller,N., Mitchell,S., Tuszyanski,A., and Russell,S.J. (1995). Tissue-specific gene expression from Mo-MLV retroviral vectors with hybrid LTRs containing the murine tyrosinase enhancer/promoter. *Virology* **214,** 307-313.

Vile,R.G. and Hart,I.R. (1993a). In vitro and in vivo targeting of gene expression to melanoma cells. *Cancer Res.* **53,** 962-967.

Vile,R.G. and Hart,I.R. (1993b). Use of tissue-specific expression of the herpes simplex virus thymidine kinase gene to inhibit growth of established murine melanomas following direct intratumoral injection of DNA. *Cancer Res.* **53,** 3860-3864.

Vile,R.G., Nelson,J.A., Castleden,S., Chong,H., and Hart,I.R. (1994b). Systemic gene therapy of murine melanoma using tissue specific expression of the HSVtk gene involves an immune component. *Cancer Res.* **54,** 6228-6234.

Vogelstein,B. and Gillespie,D. (1979). Preparative and analytical purification of DNA from agarose. *Proc. Natl. Acad. Sci. U. S. A* **76,** 615-619.

Volpp,B.D. and Lin,Y. (1993). In vitro molecular reconstitution of the respiratory burst in B lymphoblasts from p47-phox-deficient chronic granulomatous disease. *J. Clin. Invest* **91,** 201-207.

Volpp,B.D., Nauseef,W.M., and Clark,R.A. (1988). Two cytosolic neutrophil oxidase components absent in autosomal chronic granulomatous disease. *Science* **242,** 1295-1297.

Voso,M.T., Burn,T.C., Wulf,G., Lim,B., Leone,G., and Tenen,D.G. (1994). Inhibition of hematopoiesis by competitive binding of transcription factor PU.1. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7932-7936.

Vowells,S.J., Sekhsaria,S., Malech,H.L., Shalit,M., and Fleisher,T.A. (1995). Flow cytometric analysis of the granulocyte respiratory burst: a comparison study of fluorescent probes. *J. Immunol. Methods* **178,** 89-97.

Wang,S., Wang,Q., Crute,B.E., Melnikova,I.N., Keller,S.R., and Speck,N.A. (1993). Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. *Mol. Cell Biol.* **13,** 3324-3339.

Weening,R.S., Kabel,P., Pijman,P., and Roos,D. (1983). Continuous therapy with sulfamethoxazole-trimethoprim in patients with chronic granulomatous disease. *J. Pediatr.* **103,** 127-130.

Werner,M., Kraunus,J., Baum,C., and Brocker,T. (2004). B-cell-specific transgene expression using a self-inactivating retroviral vector with human CD19 promoter and viral post-transcriptional regulatory element. *Gene Ther.* **11,** 992-1000.

Wientjes,F.B., Hsuan,J.J., Totty,N.F., and Segal,A.W. (1993). p40phox, a third cytosolic component of the activation complex of the NADPH oxidase to contain src homology 3 domains. *Biochem. J.* **296 (Pt 3),** 557-561.

Wigler,M., Silverstein,S., Lee,L.S., Pellicer,A., Cheng,Y., and Axel,R. (1977). Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* **11,** 223-232.

Wilkinson,G.W. and Akrigg,A. (1992). Constitutive and enhanced expression from the CMV major IE promoter in a defective adenovirus vector. *Nucleic. Acids. Res.* **20,** 2233-2239.

Wilkinson,N.C. and Navarro,J. (1999). PU.1 regulates the CXCR1 promoter. *J. Biol. Chem.* **274,** 438-443.

References

Williams,D.A., Tao,W., Yang,F., Kim,C., Gu,Y., Mansfield,P., Levine,J.E., Petryniak,B., Derrow,C.W., Harris,C. et al. (2000). Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. *Blood* **96,** 1646-1654.

Winkelstein,J.A., Marino,M.C., Johnston,R.B., Jr., Boyle,J., Curnutte,J., Gallin,J.I., Malech,H.L., Holland,S.M., Ochs,H., Quie,P. et al. (2000). Chronic granulomatous disease. Report on a national registry of 368 patients. *Medicine (Baltimore)* **79,** 155-169.

Wolf,H., Fruhwirth,M., Ruedl,C., Oswald,H.P., Fischer,H., Bock,G., and Wick,G. (1995). Chronic granulomatous disease assessed by single-cell granulocyte oxidative burst activity. *Int. Arch. Allergy Immunol.* **106,** 425-427.

Wotton,D., Ghysdael,J., Wang,S., Speck,N.A., and Owen,M.J. (1994). Cooperative binding of Ets-1 and core binding factor to DNA. *Mol. Cell Biol.* **14,** 840-850.

Yanez-Munoz,R.J., Balaggan,K.S., MacNeil,A., Howe,S.J., Schmidt,M., Smith,A.J., Buch,P., MacLaren,R.E., Anderson,P.N., Barker,S.E. et al. (2006). Effective gene therapy with nonintegrating lentiviral vectors. *Nat. Med.* **12,** 348-353.

Yang,S., Delgado,R., King,S.R., Woffendin,C., Barker,C.S., Yang,Z.Y., Xu,L., Nolan,G.P., and Nabel,G.J. (1999). Generation of retroviral vector for clinical studies using transient transfection. *Hum. Gene Ther.* **10,** 123-132.

Yang,Z., Wara-Aswapati,N., Chen,C., Tsukada,J., and Auron,P.E. (2000). NF-IL6 (C/EBPbeta) vigorously activates il1b gene expression via a Spi-1 (PU.1) protein-protein tether. *J. Biol. Chem.* **275,** 21272-21277.

Yee,J.K., Moores,J.C., Jolly,D.J., Wolff,J.A., Respess,J.G., and Friedmann,T. (1987). Gene expression from transcriptionally disabled retroviral vectors. *Proc. Natl. Acad. Sci. U. S. A* **84,** 5197-5201.

Yi,Y., Hahm,S.H., and Lee,K.H. (2005). Retroviral gene therapy: safety issues and possible solutions. *Curr. Gene Ther.* **5,** 25-35.

Yu,L., Zhen,L., and Dinauer,M.C. (1997). Biosynthesis of the phagocyte NADPH oxidase cytochrome b558. Role of heme incorporation and heterodimer formation in maturation and stability of gp91phox and p22phox subunits. *J. Biol. Chem.* **272,** 27288-27294.

Yu,S.F., von Ruden,T., Kantoff,P.W., Garber,C., Seiberg,M., Ruther,U., Anderson,W.F., Wagner,E.F., and Gilboa,E. (1986). Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proc. Natl. Acad. Sci. U. S. A* **83,** 3194-3198.

Zhang,D.E., Fujioka,K., Hetherington,C.J., Shapiro,L.H., Chen,H.M., Look,A.T., and Tenen,D.G. (1994a). Identification of a region which directs the monocytic activity of the colony-stimulating factor 1 (macrophage colony-stimulating factor) receptor promoter and binds PEBP2/CBF (AML1). *Mol. Cell Biol.* **14,** 8085-8095.

Zhang,D.E., Hetherington,C.J., Chen,H.M., and Tenen,D.G. (1994b). The macrophage transcription factor PU.1 directs tissue-specific expression of the macrophage colony-stimulating factor receptor. *Mol. Cell Biol.* **14,** 373-381.

Zhang,D.E., Hetherington,C.J., Meyers,S., Rhoades,K.L., Larson,C.J., Chen,H.M., Hiebert,S.W., and Tenen,D.G. (1996). CCAAT enhancer-binding protein (C/EBP) and AML1 (CBF alpha2) synergistically activate the macrophage colony-stimulating factor receptor promoter. *Mol. Cell Biol.* **16,** 1231-1240.

Zhang,D.E., Hetherington,C.J., Tan,S., Dziennis,S.E., Gonzalez,D.A., Chen,H.M., and Tenen,D.G. (1994c). Sp1 is a critical factor for the monocytic specific expression of human CD14. *J. Biol. Chem.* **269,** 11425-11434.

Zhang,D.E., Zhang,P., Wang,N.D., Hetherington,C.J., Darlington,G.J., and Tenen,D.G. (1997). Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha- deficient mice. *Proc. Natl. Acad. Sci. U. S. A* **94,** 569-574.

Zhen,L., King,A.A., Xiao,Y., Chanock,S.J., Orkin,S.H., and Dinauer,M.C. (1993). Gene targeting of X chromosome-linked chronic granulomatous disease locus in a human myeloid

leukemia cell line and rescue by expression of recombinant gp91phox. *Proc. Natl. Acad. Sci. U. S. A* **90,** 9832-9836.

Zhu,J. and Emerson,S.G. (2002). Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene* **21,** 3295-3313.

Zufferey,R., Donello,J.E., Trono,D., and Hope,T.J. (1999). Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73,** 2886-2892.

8 Appendix: Plasmid Maps and Cloning Strategies

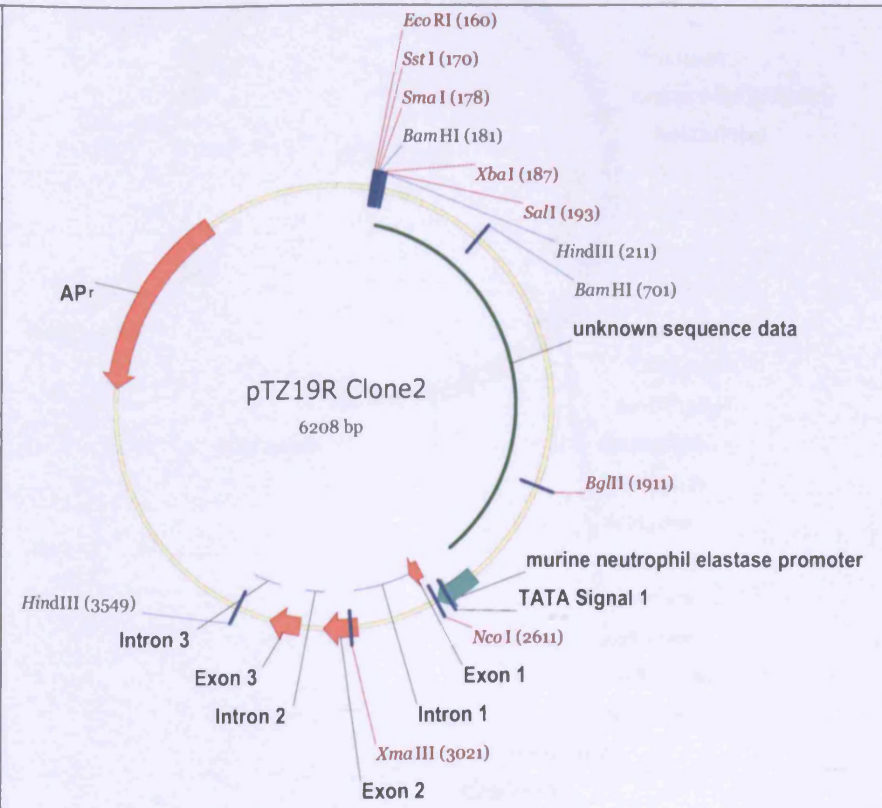
The following list of plasmid restriction maps were drawn up with the help of the VectorNTI DNA sequence database software from InforMax, Inc. (Invitrogen) to represent each plasmid, subclones and cloning strategies used in this study. Restriction maps were based on the DNA sequence obtained from GenBank NCBI database or where no sequence data was available the known base pair length and restriction site positions of the DNA fragment are indicated. Unique restriction sites are printed in red, while other restriction sites are printed in black. Promoter direction, gene coding sequences (cds) and reading frame orientation are indicated by an arrow, whereas transcription factor binding sites, IRES, exon and intron, ori, poly-A signal locations are specified at their known positions.

8.1 Plasmids with origin of promoters for investigation

8.1.1 pTZ19R clone2 (murine neutrophile elastase promoter)

Promoter: murine neutrophile elastase (~2400bp)

Reference: (Nuchprayoon et al., 1994)

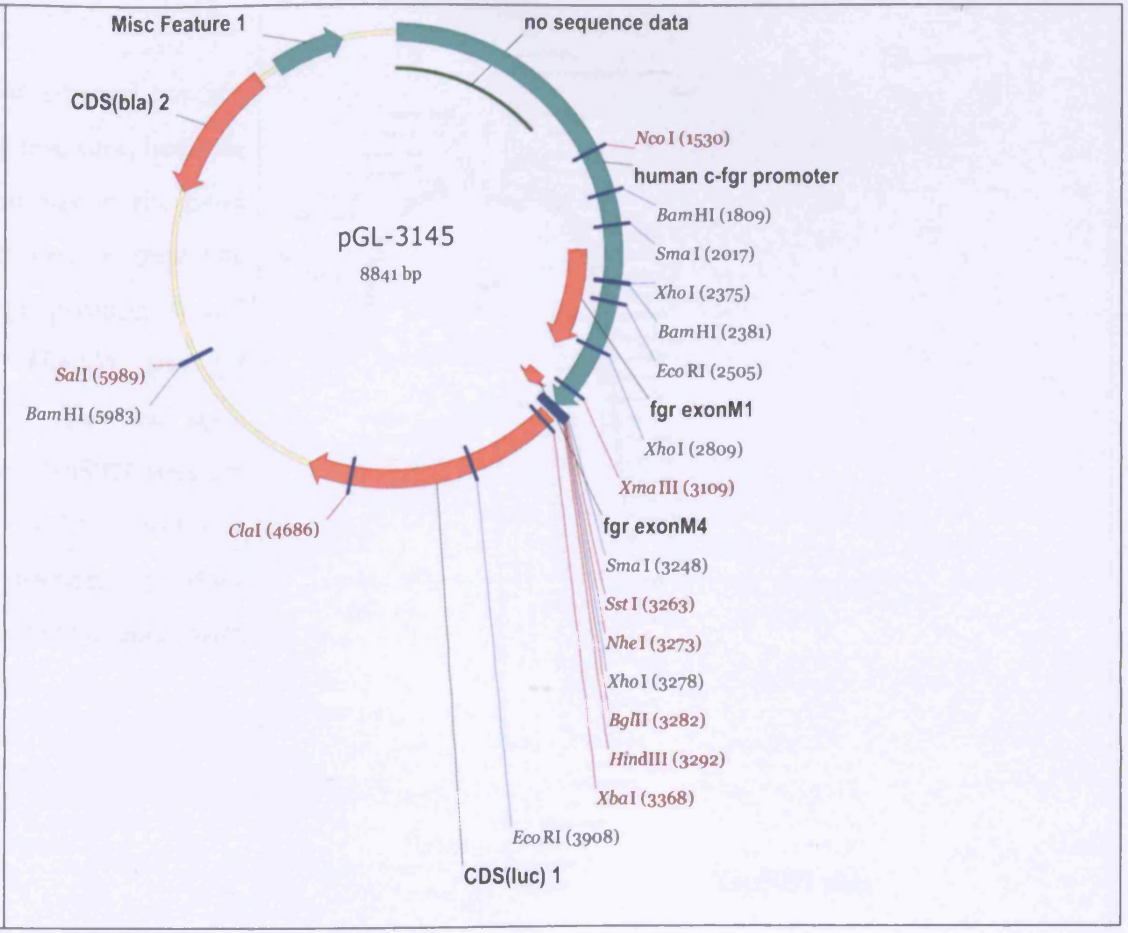


8.1.2 pGL-3145 (human c-fgr promoter)

Promoter: c-fgr

Backbone: pGL2-Basic

Reference: (Kefalas et al., 1995)

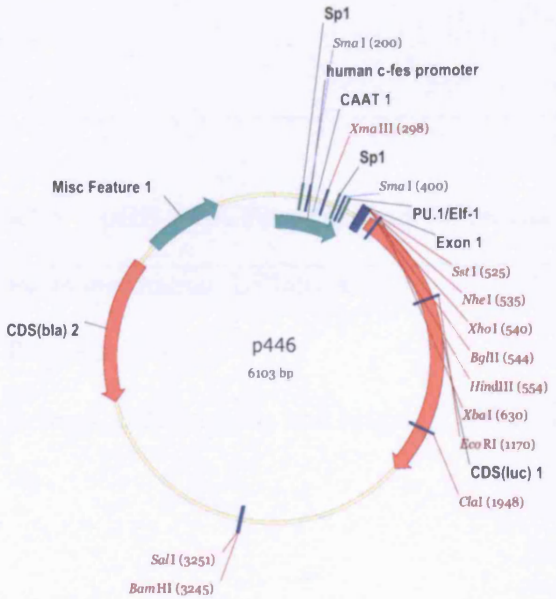


8.1.3 p446 (human c-fes promoter)

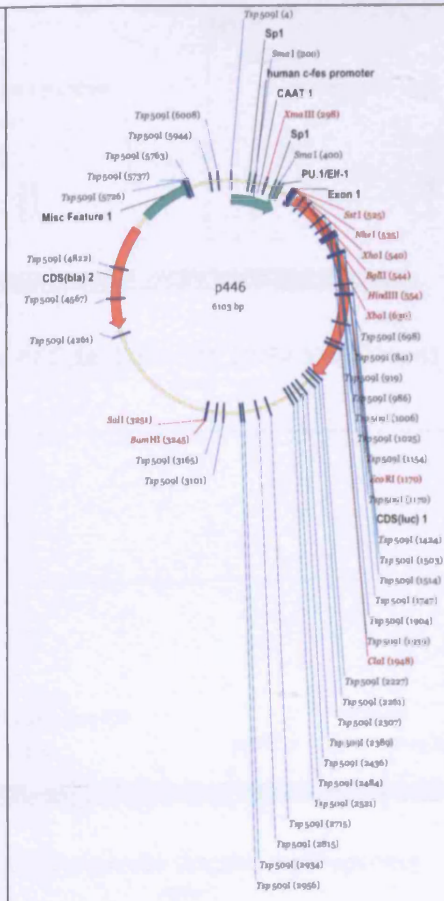
Promoter: human c-fes

Reporter gene: luciferase

Reference: (Heydemann et al., 1996; Heydemann et al., 1997; Heydemann et al., 2000)

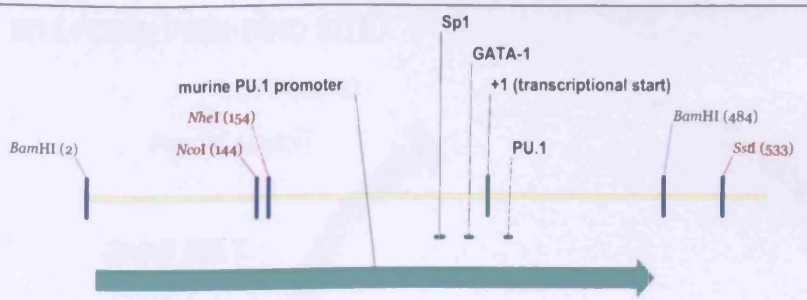


Note: the p446 plasmid has 36x *Tsp*509I restriction sites, however the c-fes promoter in the p446 plasmid is flanked by only one *Tsp*509I site (at position 4) and one unique *Hind*III site (at position 554) restriction sites, while all other Tsp509I sites are outside the c-fes promoter. *Tsp*509I restriction produce compatible cohesive ends with *Eco*RI.

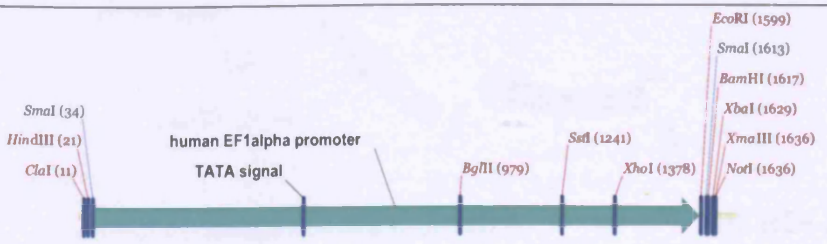


Tsp509I sites

8.1.4 pmPU.1-334/luc (murine PU.1 promoter)

Promoter: murine PU.1 Reporter gene: luciferase Reference: (Chen et al., 1995a)	 murine PU.1 (Spi-1) promoter (pmPU-334/luc insert) 569 bp

8.1.5 pEF-PGK-Neo (human EF1alpha promoter)

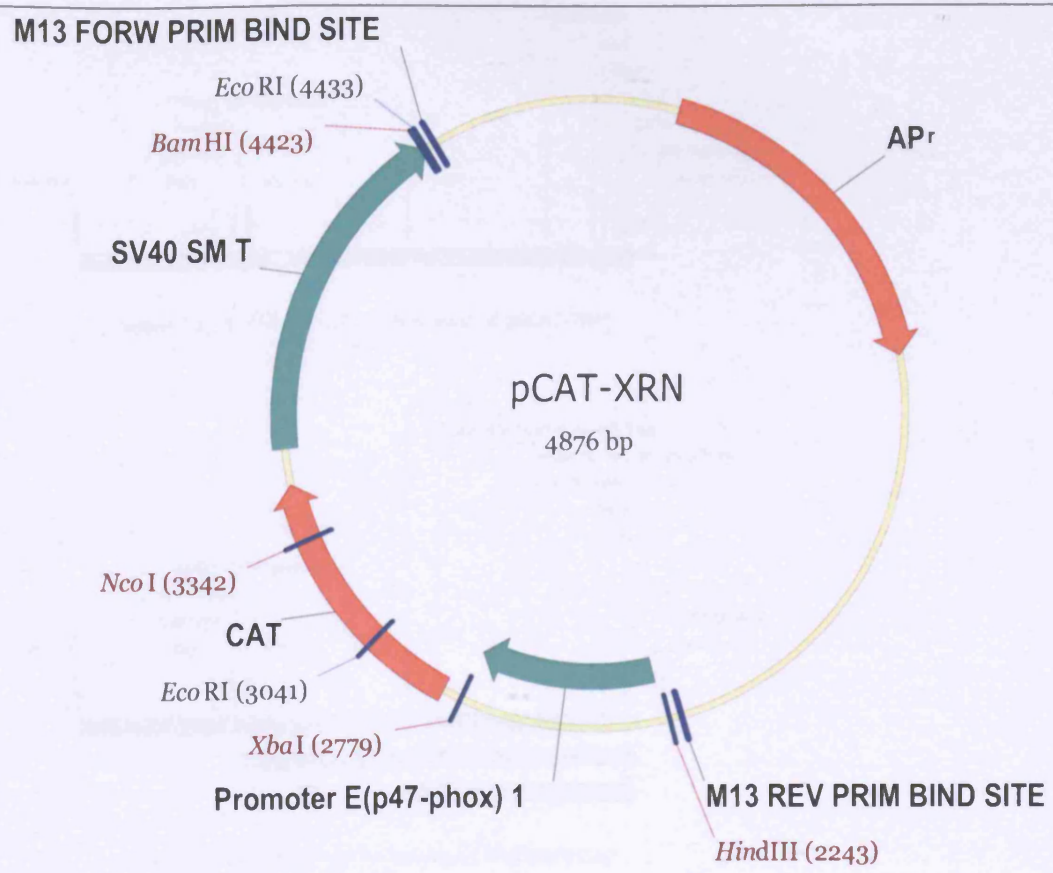
Promoter: human EF1alpha Reporter gene: Reference:(Mizushima and Nagata, 1990; Zhen et al., 1993)	 human EF1alpha promoter (fragment in pEF-pgK-Neo) 1688 bp

8.1.6 pCAT-XRN (human p47phox promoter)

Promoter: p47phox

Reporter gene: CAT

Reference: (Marden et al., 2003a; Marden et al., 2003b)

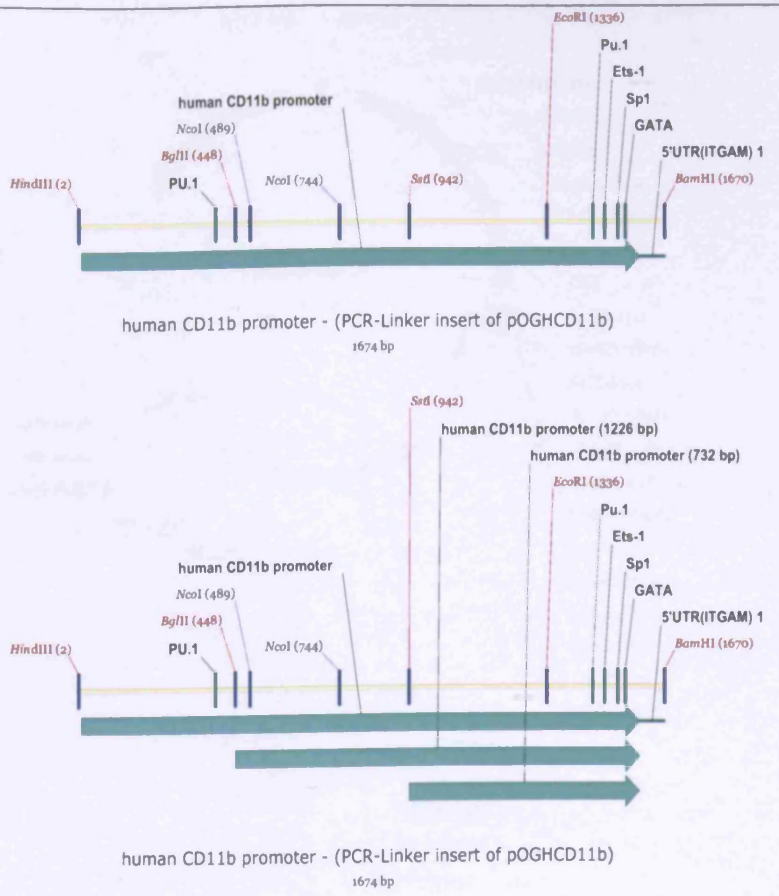


8.1.7 pΦGH-CD11b (human CD11b promoter)

Promoter: CD11b

Reporter gene:

Reference: (Hickstein et al., 1992; Pahl et al., 1992)

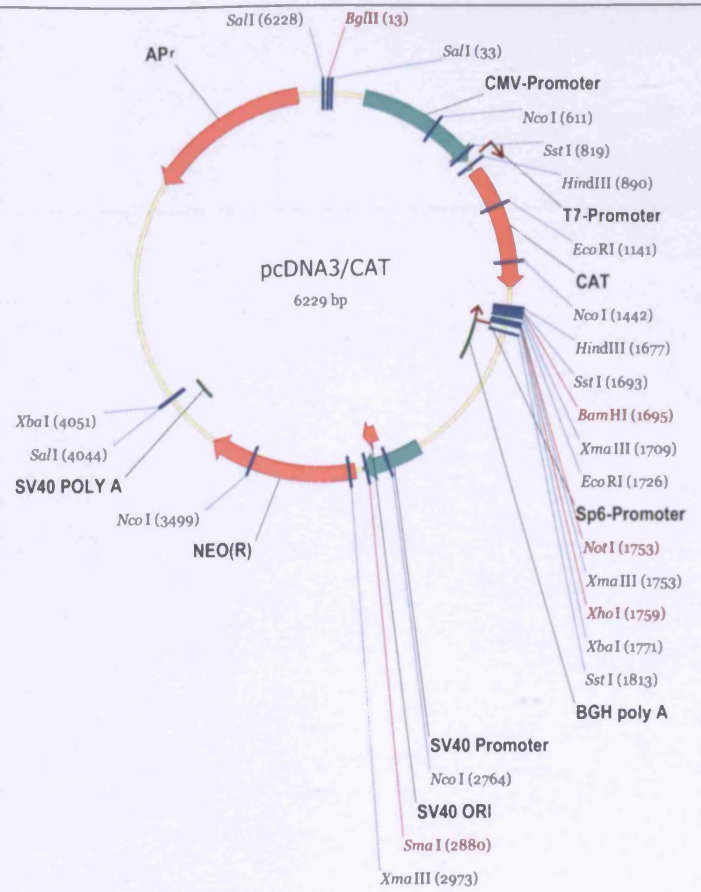


8.1.8 pcDNA3/CAT (CMV/T7 promoter)

Promoter: CMV/T7

Reporter gene: CAT

Reference: Invitrogen



8.1.9 pATCMVIXCAT3 (CMV IE promoter)

Promoter: CMV IE (~850bp) Reporter gene: CAT Reference: n/a	

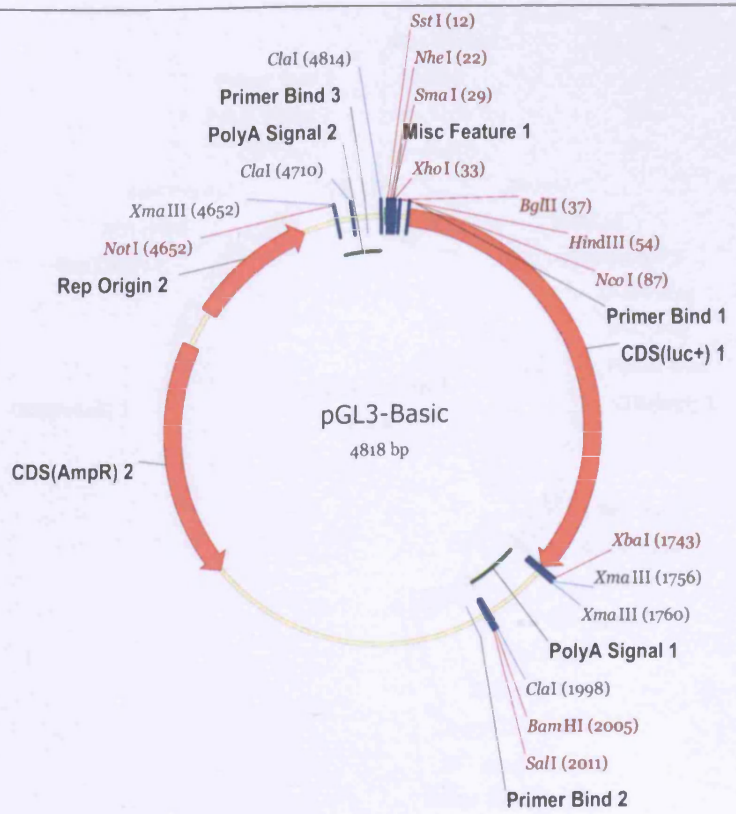
8.2 Plasmids based on pGL3-Basic with the luciferase reporter gene

8.2.1 pGL3-Basic (without promoter)

Promoter: no promoter

Reporter gene: luciferase

Reference: Promega



8.2.2 pGL3-Control (SV40 promoter)

Equal to pGL3-SV40-luc

Promoter P1: SV40 promoter

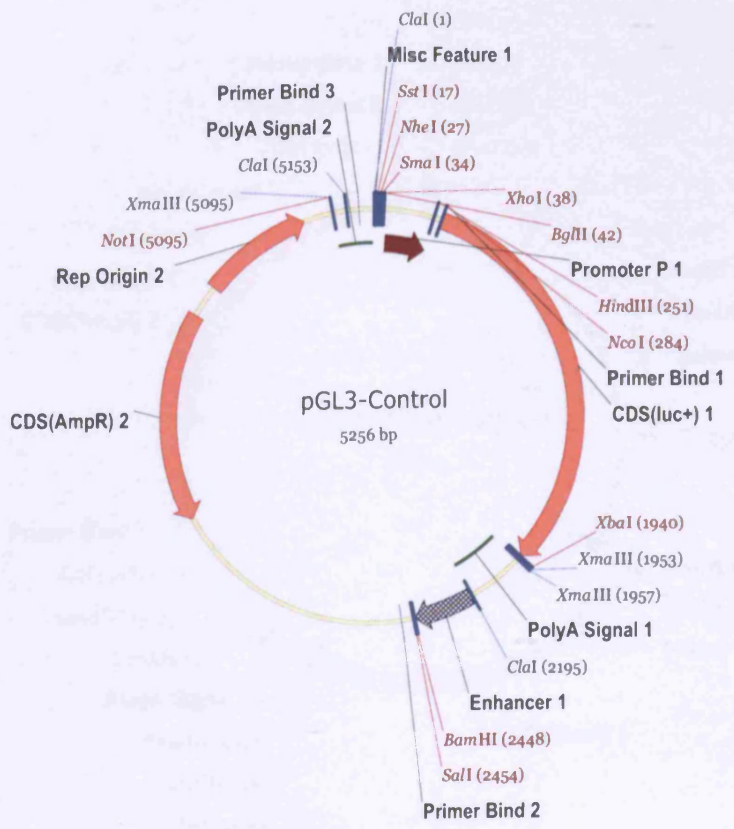
Reporter gene: CDS(luc+) luciferase

PolyA Signal 1: SV40 late ploly(A) signal

Enhancer: SV40 Enhancer

Rep Origin 2: F1 ori

Reference: Promega



8.2.3 pGL3-2400mNE-luc

Promoter: murine neutrophile elastase (~2400bp)

Reporter gene: luciferase

Cloning Strategy:

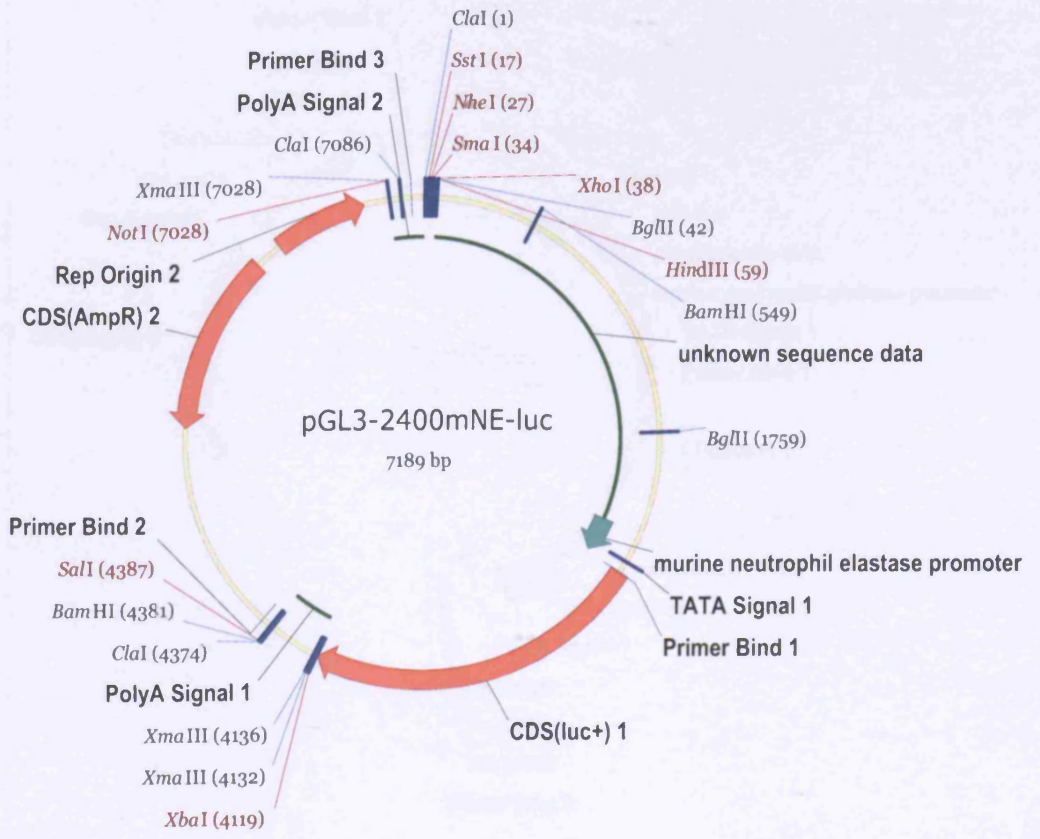
Backbone plasmid: pGL3-Basic

Digest: HindIII/NcoI

Insert plasmid: pTZ19Rclone

Digest: HindIII/NcoI

Reference: this thesis



8.2.4 pGL3-700mNE-luc

Promoter: murine neutrophile elastase

Reporter gene: luciferase

Cloning strategy:

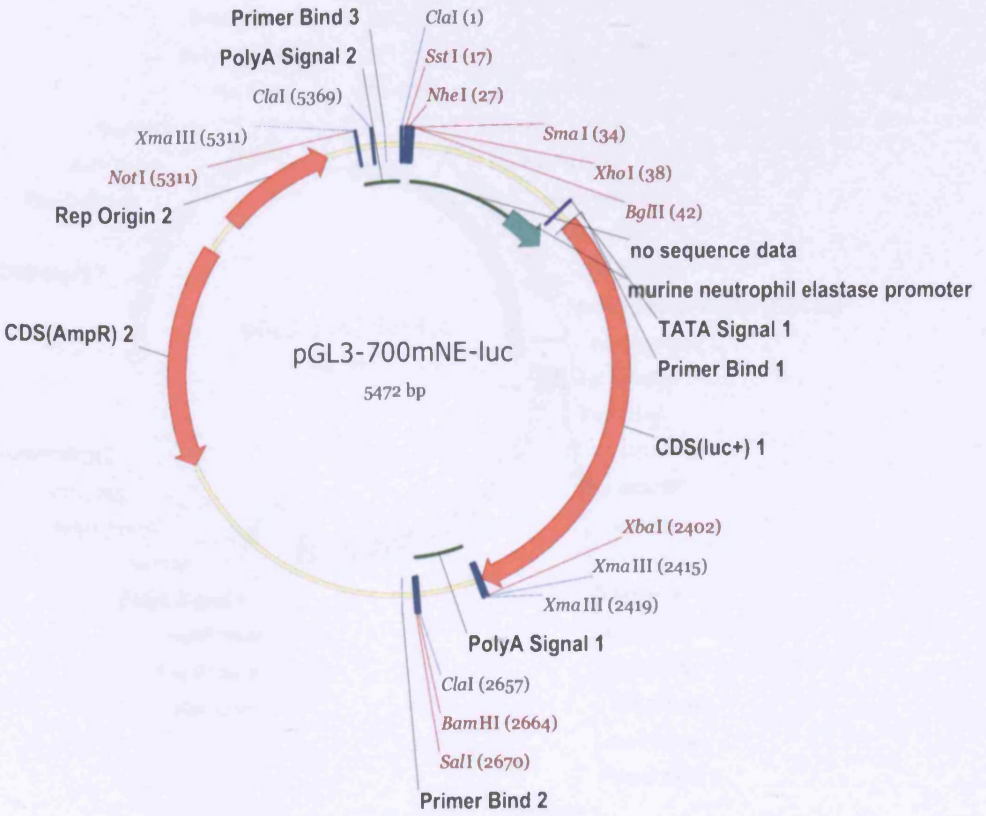
Backbone plasmid: pGL3-Basic

Digest: BglII/NcoI

Insert plasmid: pTZ19Rclone2

Digest: BglII/NcoI

Reference: this thesis



8.2.5 pGL3-1762cfgr-luc

Promoter: c-fgr (1762bp)

Reporter gene: luciferase

Cloning strategy:

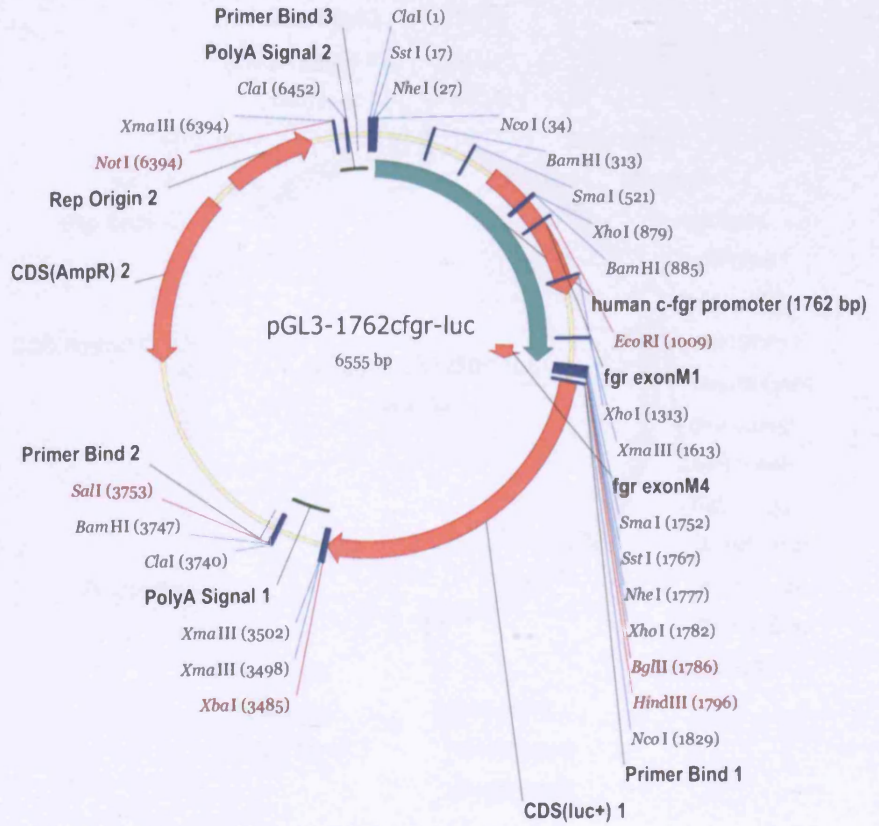
Backbone plasmid: pGL3-Basic

Digest: SmaI/HindIII

Insert plasmid: pGL-3145

Digest: NcoI (blunt)/HindIII

Reference: this thesis



8.2.6 pGL3-1231c-fgr-luc

Promoter: c-fgr (1231bp)

Reporter gene: luciferase

Cloning strategy:

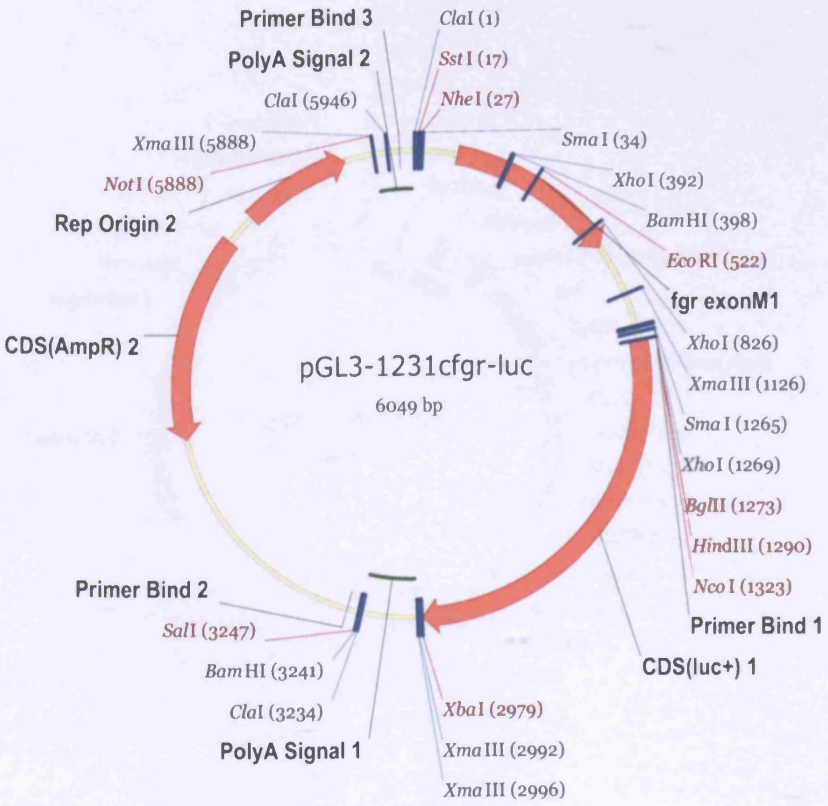
Backbone plasmid: pGL3-Basic

Digest: SmaI

Insert plasmid: pGL-3145

Digest: SmaI

Reference: this thesis



8.2.7 pGL3-mPU.1-luc

Promoter: murine PU.1

Reporter gene: luciferase

Cloning strategy:

Backbone plasmid: pGL3-Basic

Digest: BglII

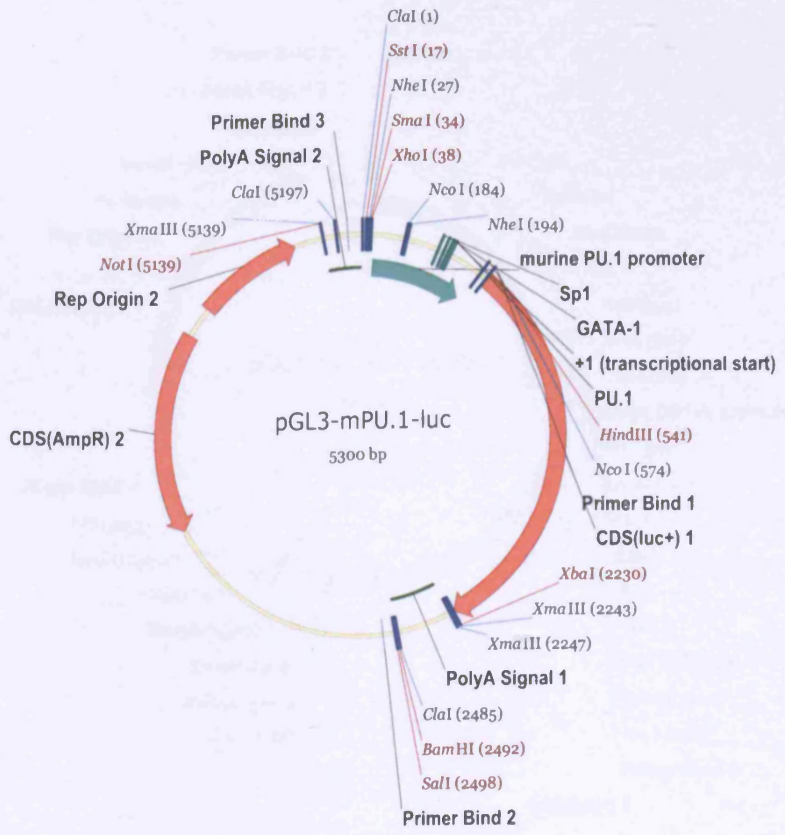
Insert plasmid: pmPU.1-334/luc

Digest: BamHI

Insert orientation confirmation

Digest: NheI and NcoI

Reference: this thesis



8.2.8 pGL3-1672CD11b-luc

Promoter: CD11b (1672bp)

Reporter gene: luciferase

Cloning strategy:

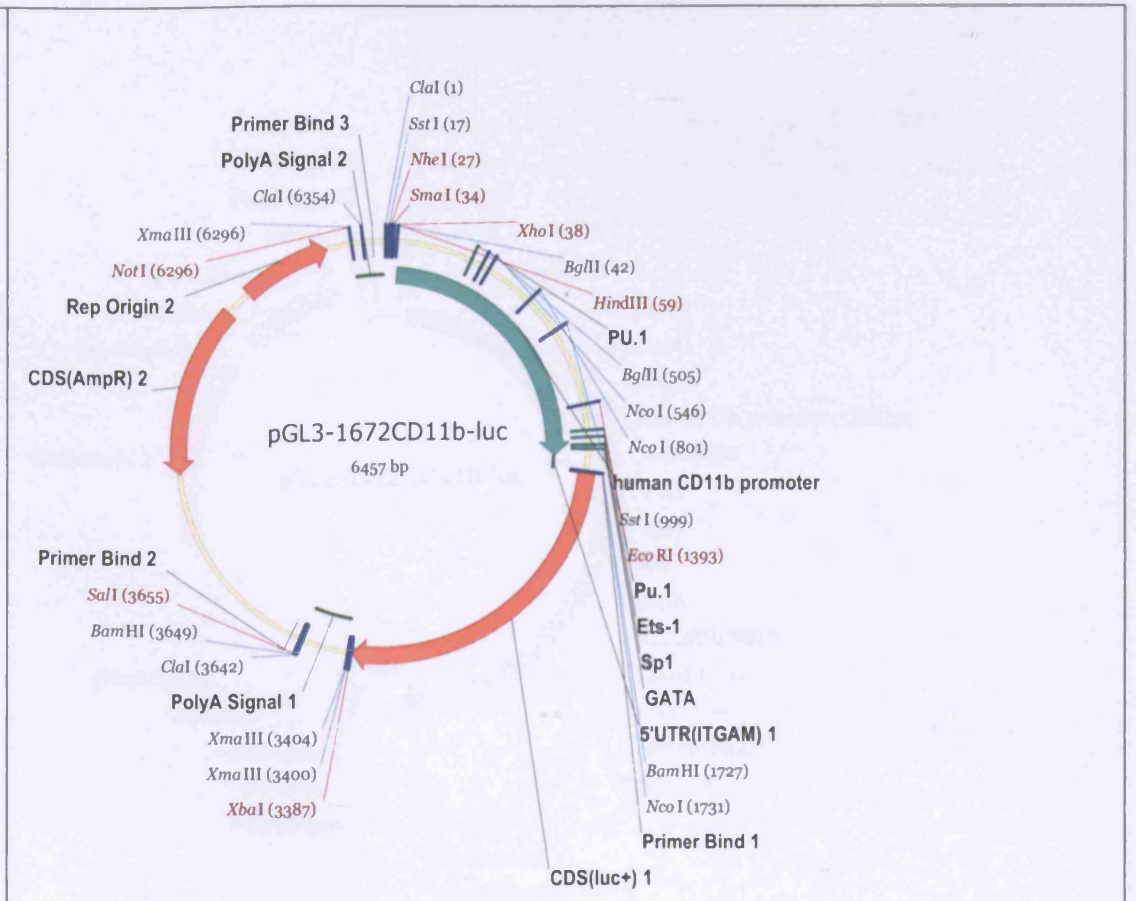
Backbone plasmid: pGL3-Basic

Digest: HindIII/NcoI(blunt)

Insert plasmid: pΦGH-CD11b

Digest: BamHI(blunt)/HindIII

Reference: this thesis



8.2.9 pGL3-1226CD11b-luc

Promoter: CD11b (1226bp)

Reporter gene: luciferase

Cloning strategy:

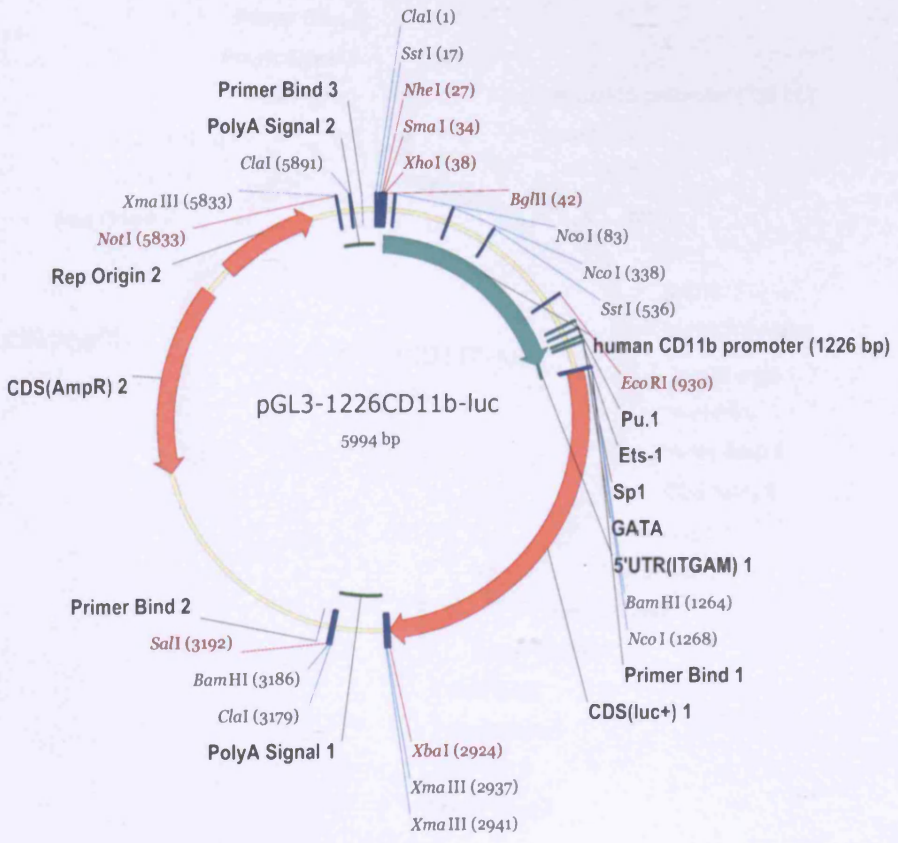
Backbone plasmid: pGL3-1672CD11b

Digest: BglII

Deletion: 464bp

Religation

Reference: this thesis



8.2.10 pGL3-732CD11b-luc

Promoter: CD11b (732bp)

Reporter gene: luciferase

Cloning strategy:

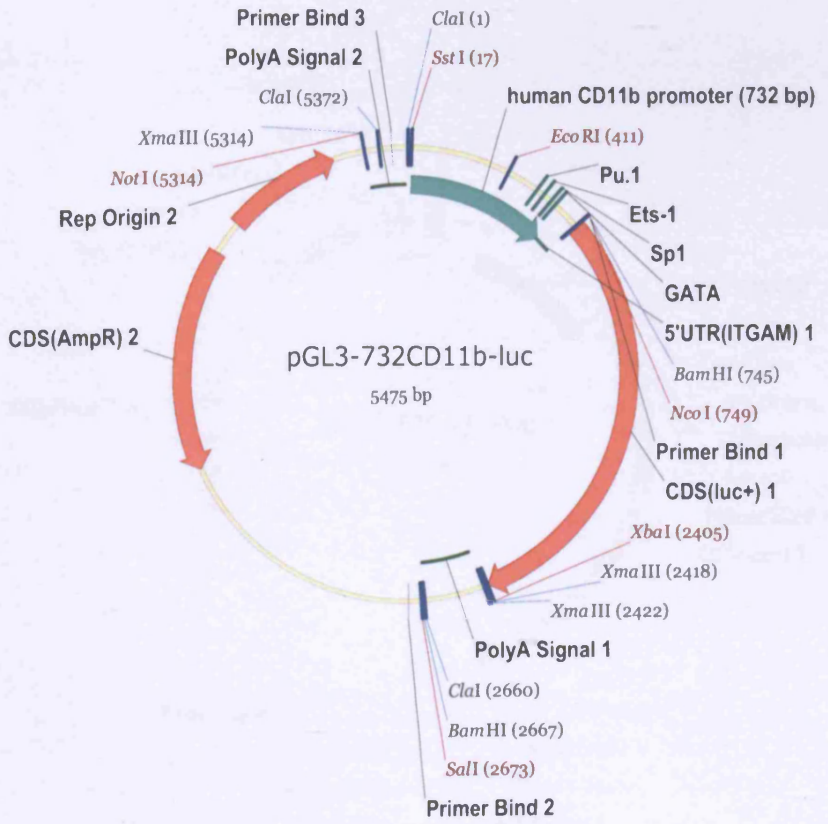
Backbone plasmid: pGL3-1672CD11b

Digest: SstI

Deletion: 982bp

Religation

Reference: this thesis



8.2.11 pGL3-CMV/T7-luc

Promoter: CMV

Reporter gene: luciferase

Cloning strategy:

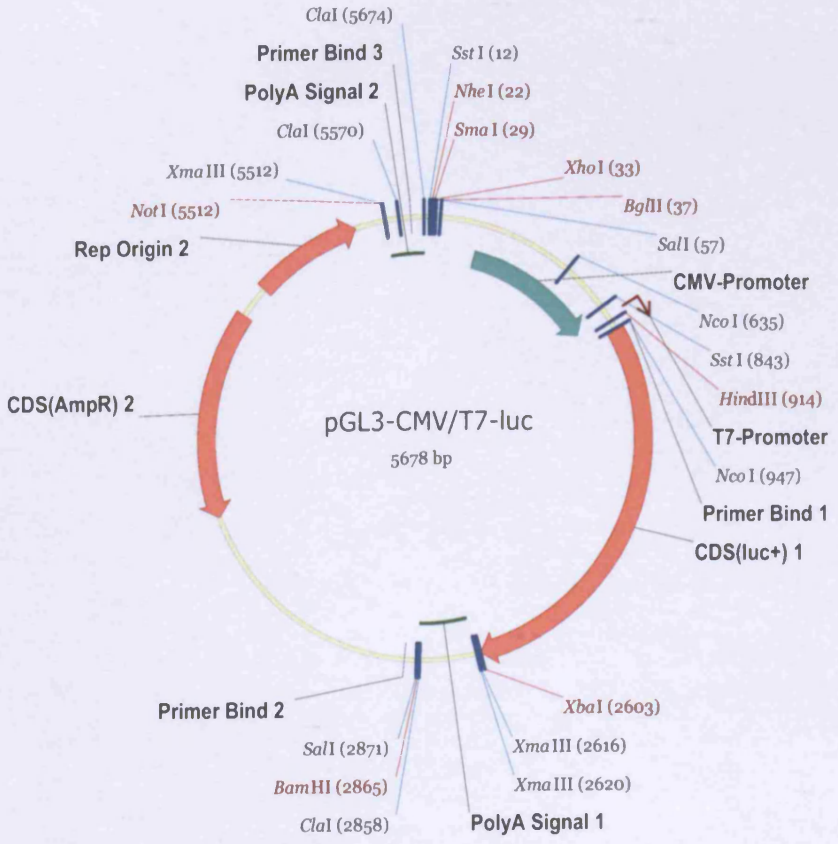
Backbone plasmid: pGL3-Basic

Digest: BglII/HindIII

Insert plasmid: pcDNA3/CAT

Digest: BglII/HindIII

Reference: this thesis



8.2.12 pGL3-CMV/T7-eGFP

Promoter: CMV

Reporter gene: eGFP

Cloning strategy:

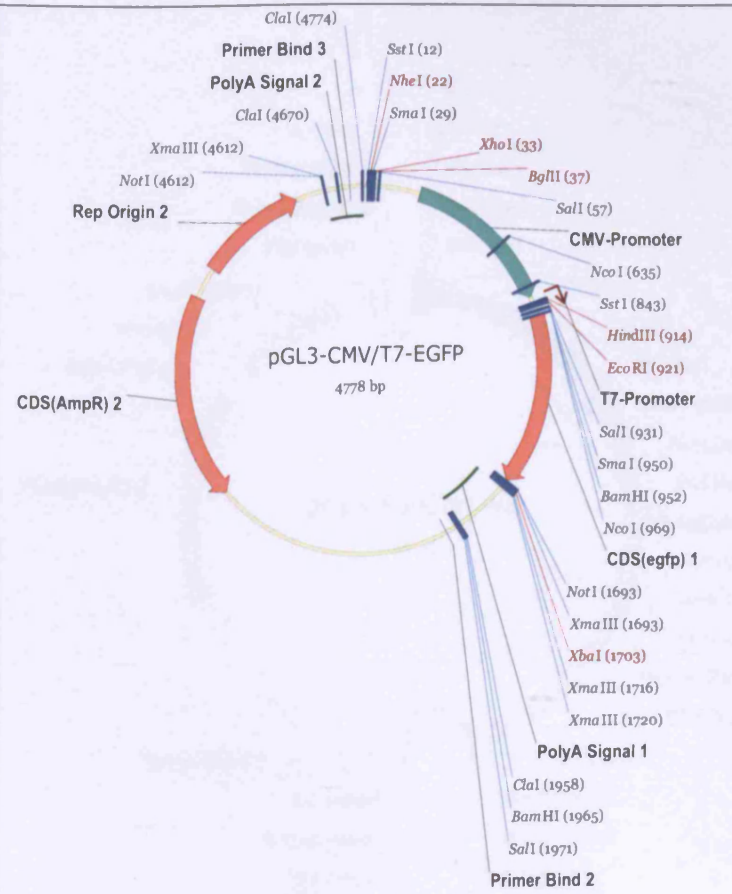
Backbone plasmid: pGL3-CMV/T7-luc

Digest: HindIII/XbaI

Insert plasmid: pEGFP-N1

Digest: HindIII/XbaI

Reference: this thesis



8.2.13 pGL3-850CMV-luc

Promoter: CMV IE (~850bp)

3'end of CMV possibly includes IntronX (no sequence data)

Reporter gene: luciferase

Cloning strategy:

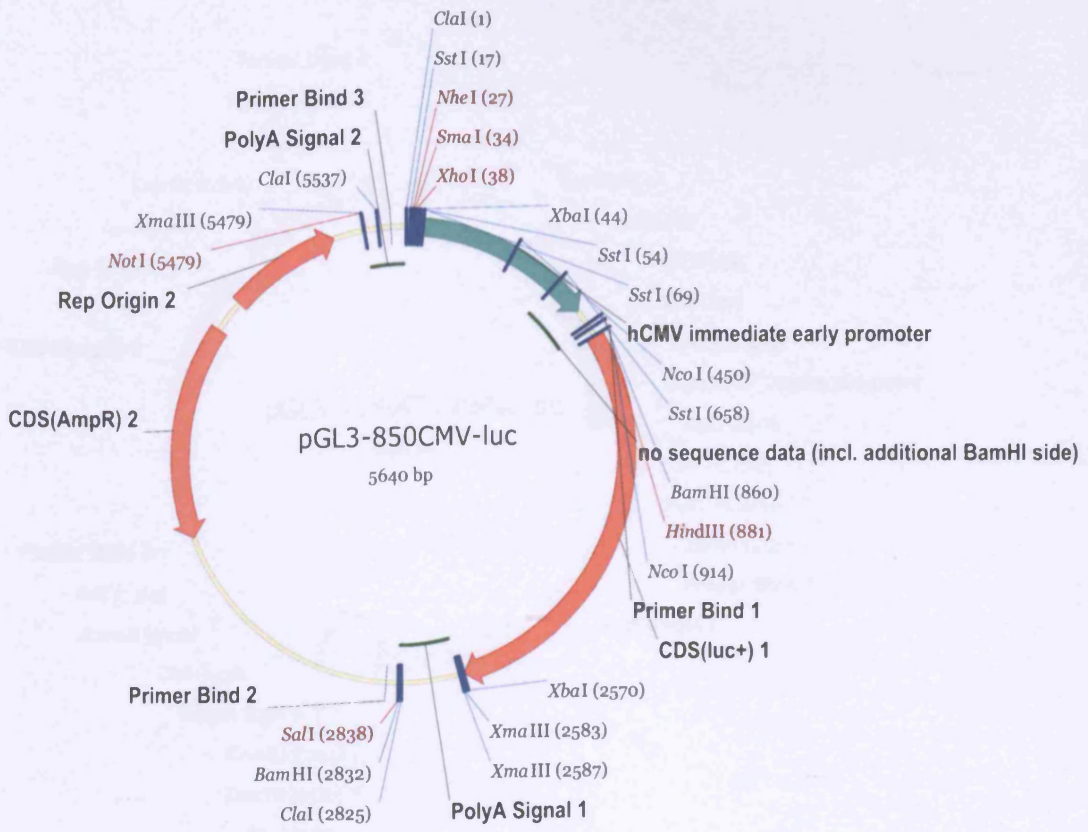
Backbone plasmid: pGL3-Basic

Digest: XhoI/HindIII

Insert plasmid: pAICMVIXCAT.3

Digest: XhoI/HindIII

Reference: this thesis



8.2.14 pGL3-1189EF-1α-luc

Promoter: EF-1α (1189bp)

Reporter gene: luciferase

Cloning strategy:

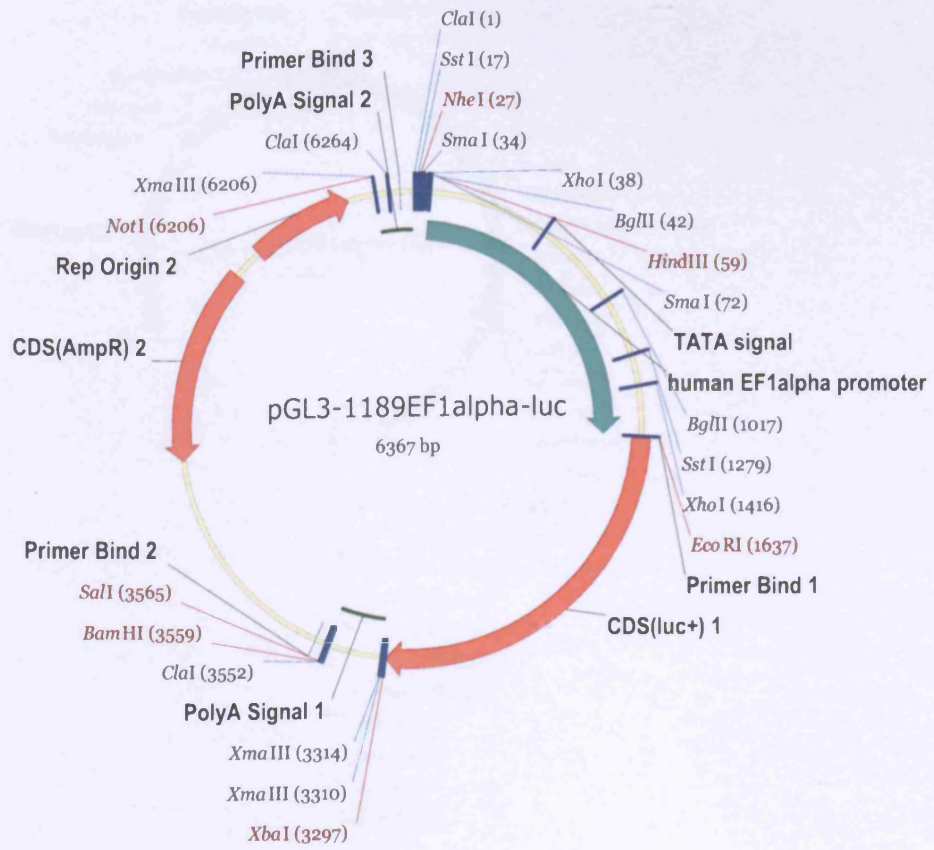
Backbone plasmid: pGL3-Basic

Digest: NcoI(blunt)/HindIII

Insert plasmid: pEF-PGK-Neo

Digest: EcoRI/HindIII

Reference: this thesis



8.2.15 pGL3-1189EF-1α-eGFP

Promoter: EF-1α (1189bp)

Reporter gene: eGFP

Cloning strategy:

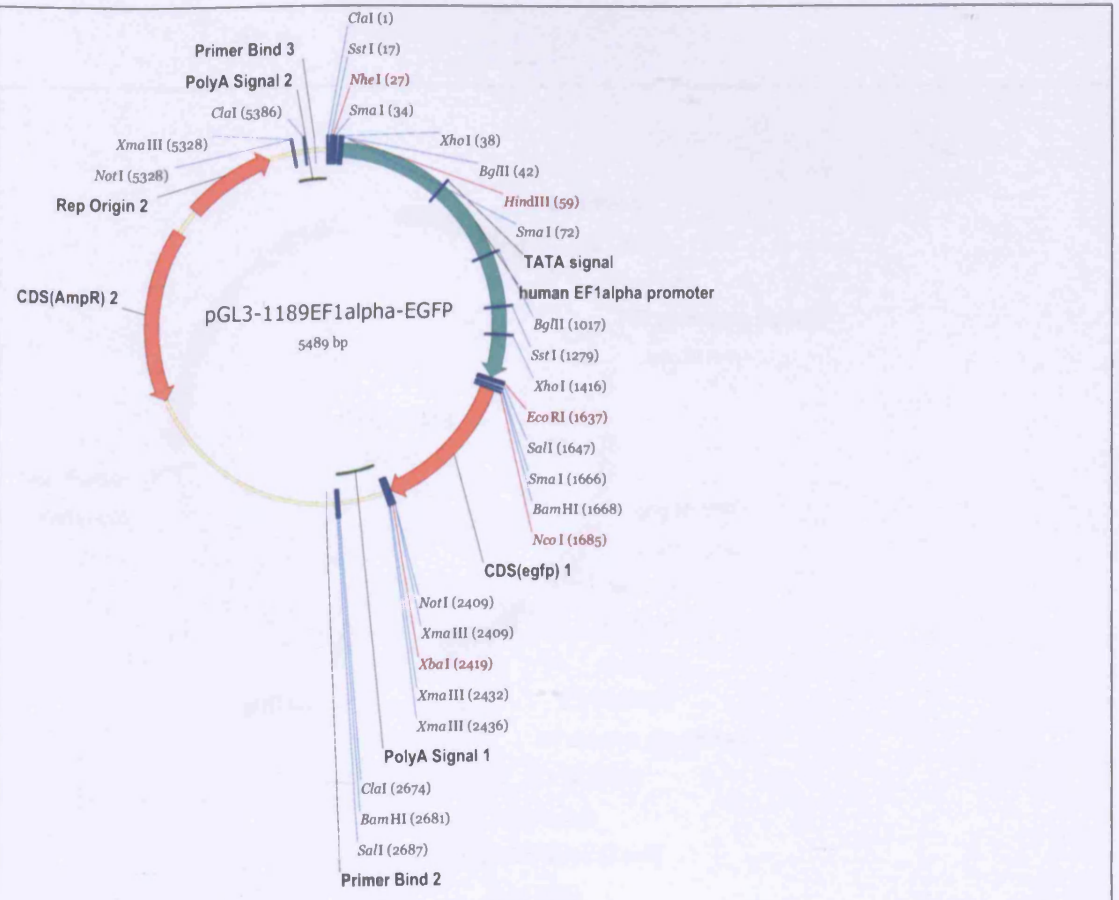
Backbone plasmid: pGL3-EF1α-luc

Digest: EcoRI/XbaI

Insert plasmid: pEGFP-N1

Digest: EcoRI/XbaI

Reference: this thesis

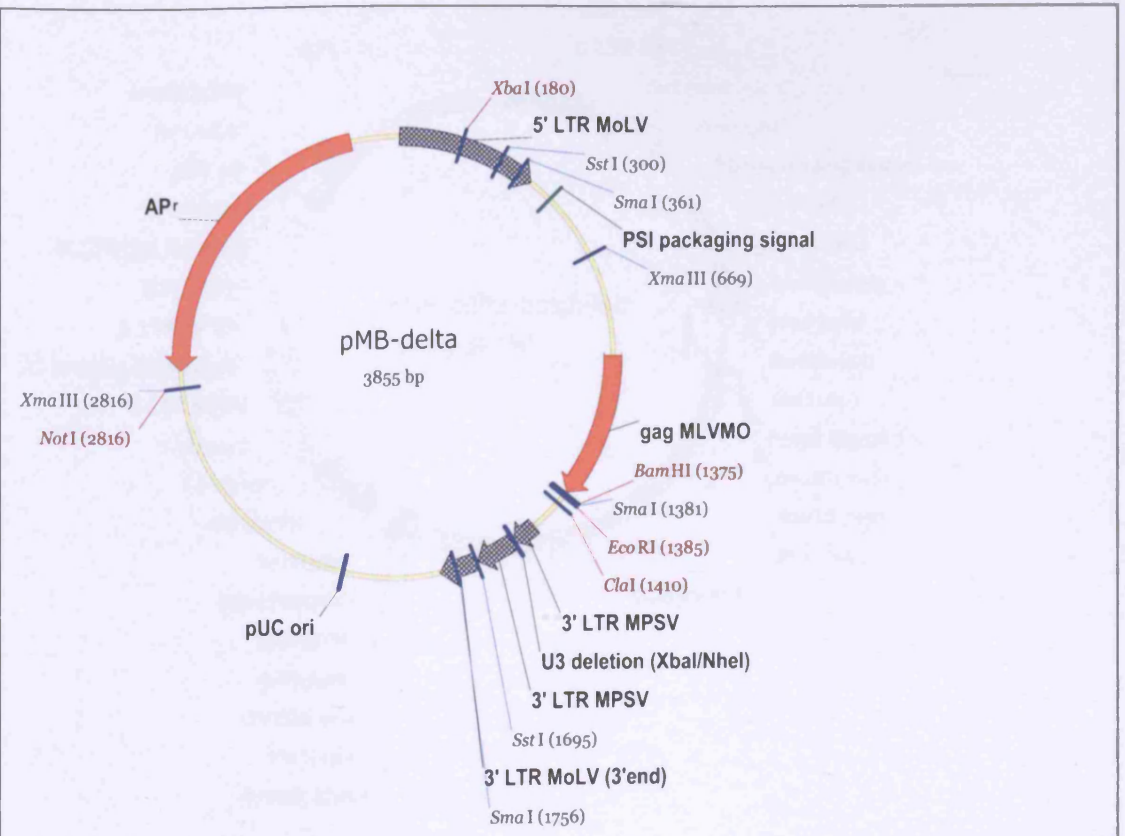


8.3 Retroviral Vectors based on pMBΔ

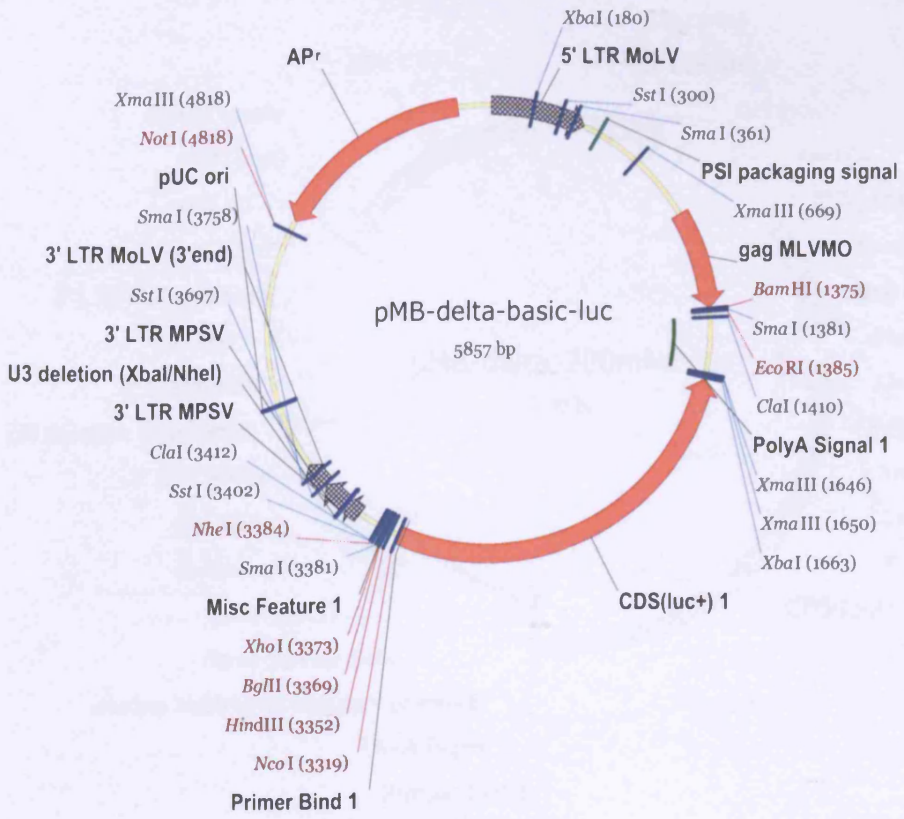
8.3.1 pMBΔ

Derivate of pBabe (Morgenstern and Land, 1990) (5'LTR: 1-1375 and 1965-4125) and pMPSV (1375-1965: 3'LTRΔ)

Reference: kind gift of Dr. C. Porter, Chester Beatty Laboratories, London



8.3.2 pMBΔ-Basic-luc

Endogenous Promoter: 5'LTR Exogenous Promoter: no Modification: enhancer deleted 3'LTR Reporter gene: luciferase Cloning strategy: Backbone plasmid: pMBΔ Digest: ClaI Insert plasmid: pGL3-Basic Digest: ClaI Insert orientation confirmation Digest: XbaI Reference: this thesis	

8.3.3 pMBΔ-700mNE-luc

Endogenous Promoter: 5'LTR

Exogenous Promoter: mNE (~700bp)

Modification: enhancer deleted 3'LTR

Reporter gene: luciferase

Cloning strategy:

Backbone plasmid: pMBΔ

Digest: BamHI

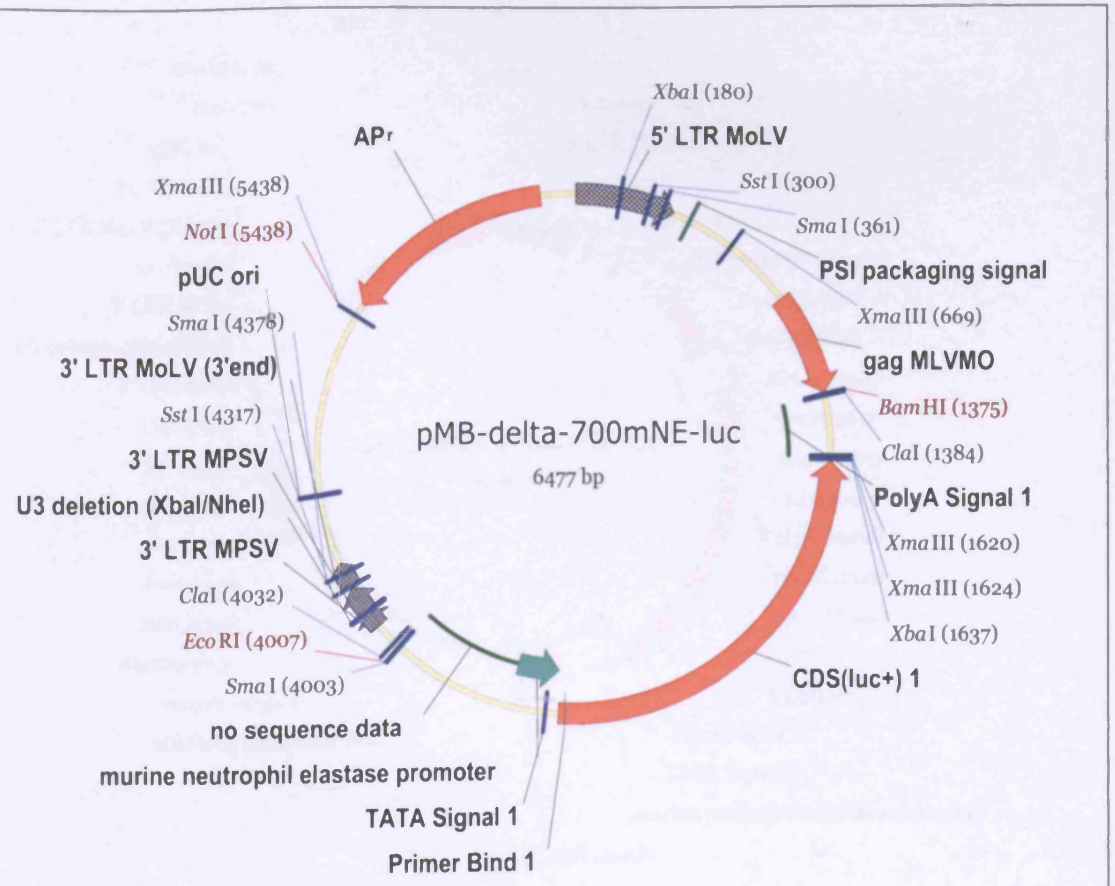
Insert plasmid: pGL3-2400mNE-luc

Digest: BglII/BamHI

Insert orientation confirmation

Digest: XbaI

Reference: this thesis



8.3.4 pMBΔ-2400NE-luc

Endogenous Promoter: 5'LTR

Exogenous Promoter: mNE (~2400bp)

Modification: enhancer deleted 3'LTR

Reporter gene: luciferase

Cloning strategy:

Backbone plasmid: pMBΔ

Digest: ClaI

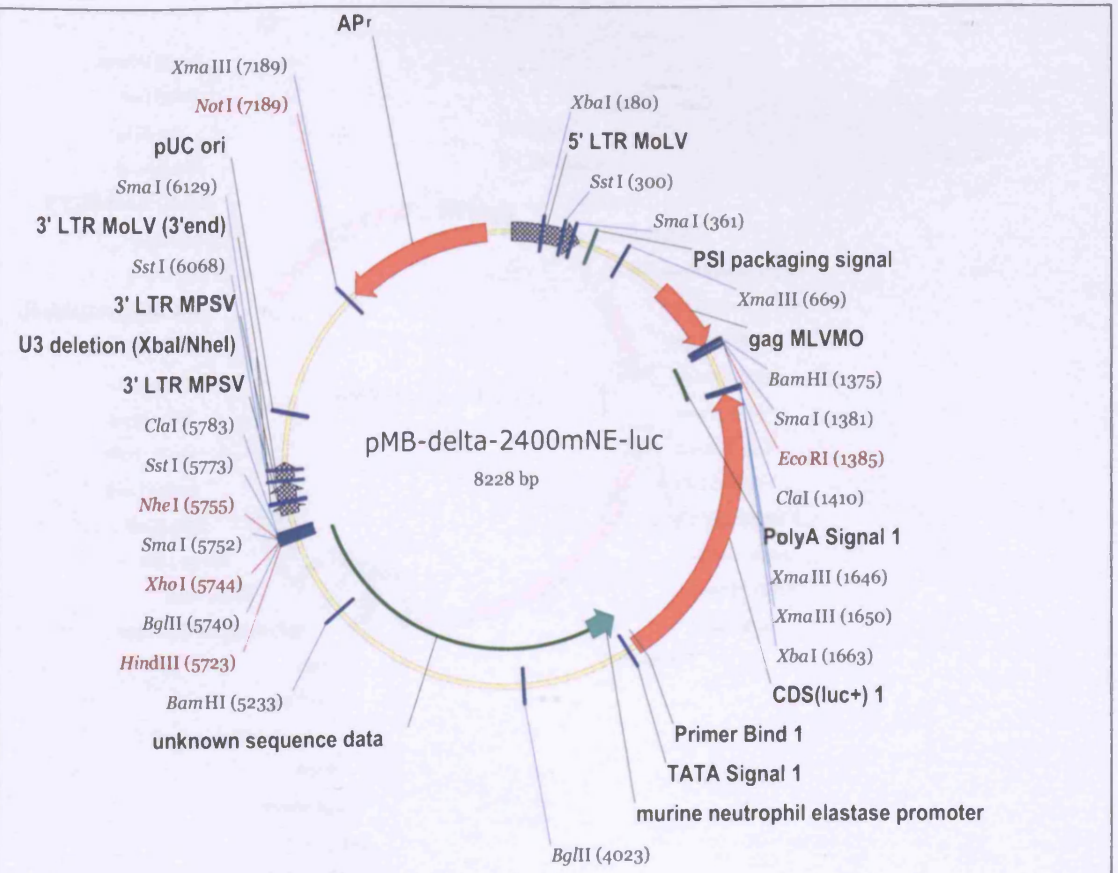
Insert plasmid: pGL3-2400mNE-luc

Digest: ClaI

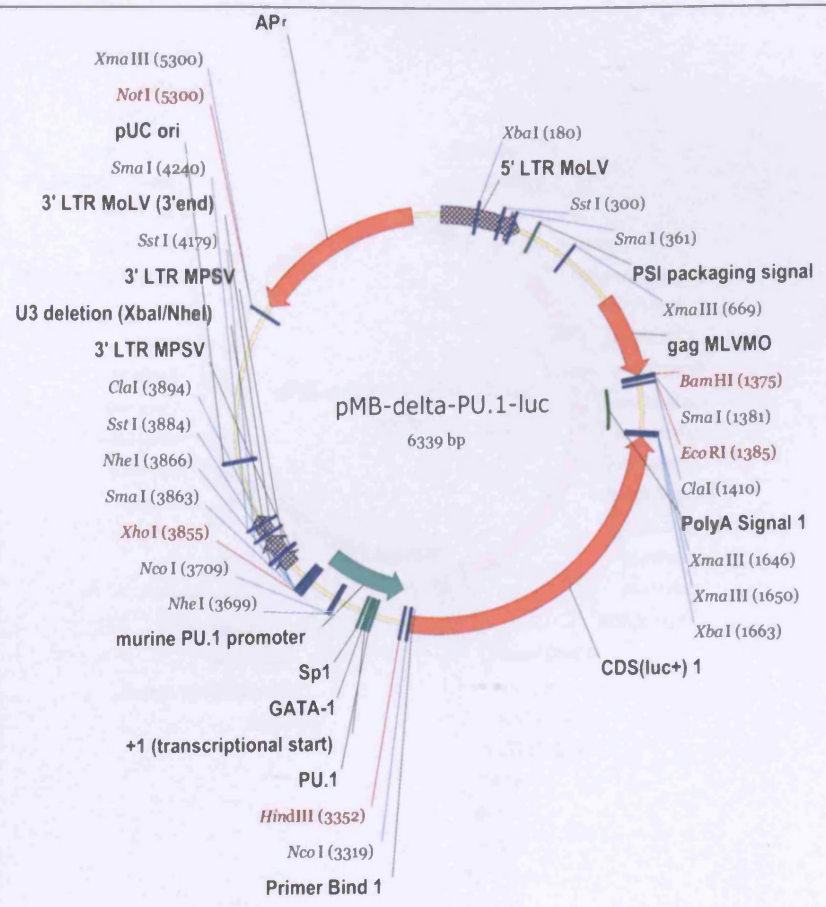
Insert orientation confirmation

Digest: XbaI

Reference: this thesis



8.3.5 pMBΔ-PU.1-luc

Endogenous Promoter: 5'LTR Exogenous Promoter: PU.1 Modification: enhancer deleted 3'LTR Reporter gene: luciferase Cloning strategy: Backbone plasmid: pMBΔ Digest: ClaI Insert plasmid: pGL3-PU.1-luc Digest: ClaI Insert orientation confirmation Digest: XbaI Reference: this thesis	

8.3.6 pMBΔ-1672CD11b-luc

Endogenous Promoter: 5'LTR

Exogenous Promoter: CD11b (1672bp)

Modification: enhancer deleted 3'LTR

Reporter gene: luciferase

Cloning strategy:

Backbone plasmid: pMBΔ

Digest: ClaI

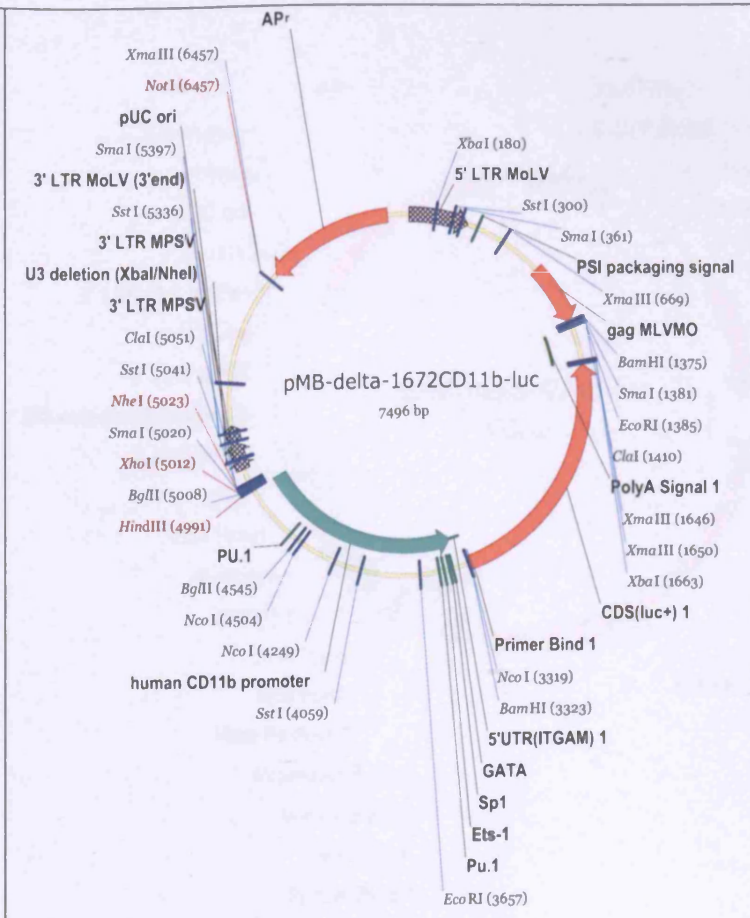
Insert plasmid: pGL3-1672CD11b-luc

Digest: ClaI

Insert orientation confirmation

Digest: XbaI

Reference: this thesis



8.3.7 pMBΔ-SV40-luc

Endogenous Promoter: 5'LTR

Exogenous Promoter: SV40

Modification: enhancer deleted 3'LTR

Reporter gene: luciferase

Cloning strategy:

Backbone plasmid: pMBΔ

Digest: ClaI

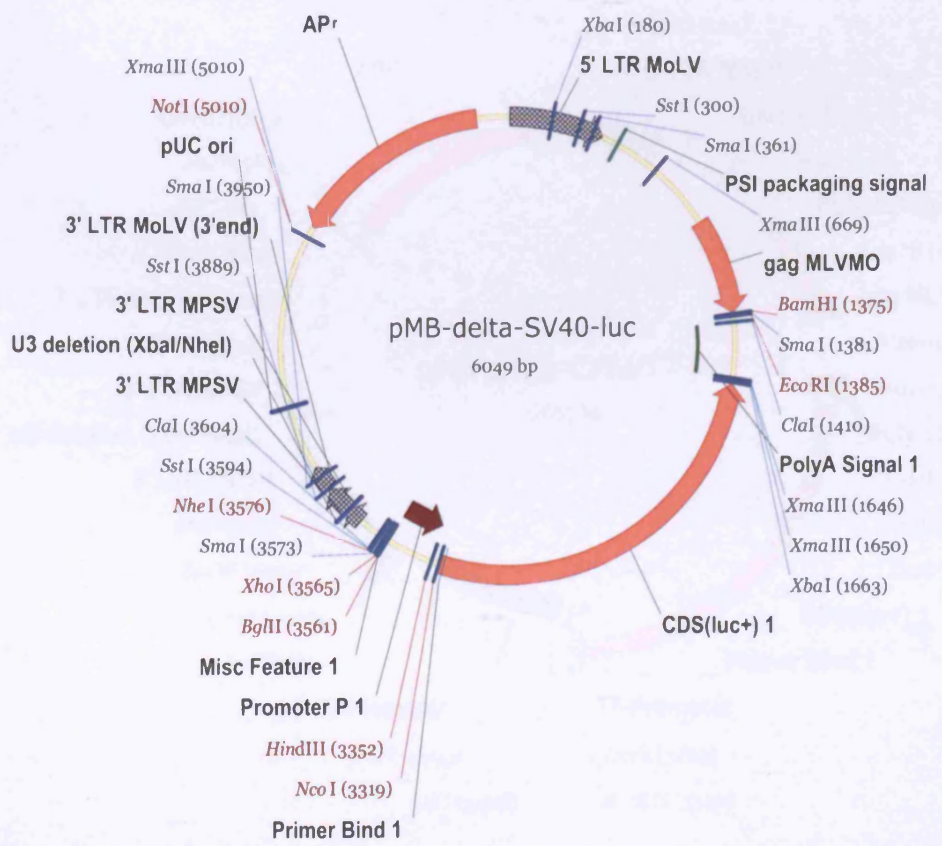
Insert plasmid: pGL3-Control (= pGL3-SV40-luc)

Digest: ClaI

Insert orientation confirmation

Digest: XbaI

Reference: this thesis



8.3.8 pMBΔ-CMV/T7-luc

Endogenous Promoter: 5'LTR

Exogenous Promoter: CMV/T7

Modification: enhancer deleted 3'LTR

Reporter gene: luciferase

Cloning strategy:

Backbone plasmid: pMBΔ

Digest: BamHI

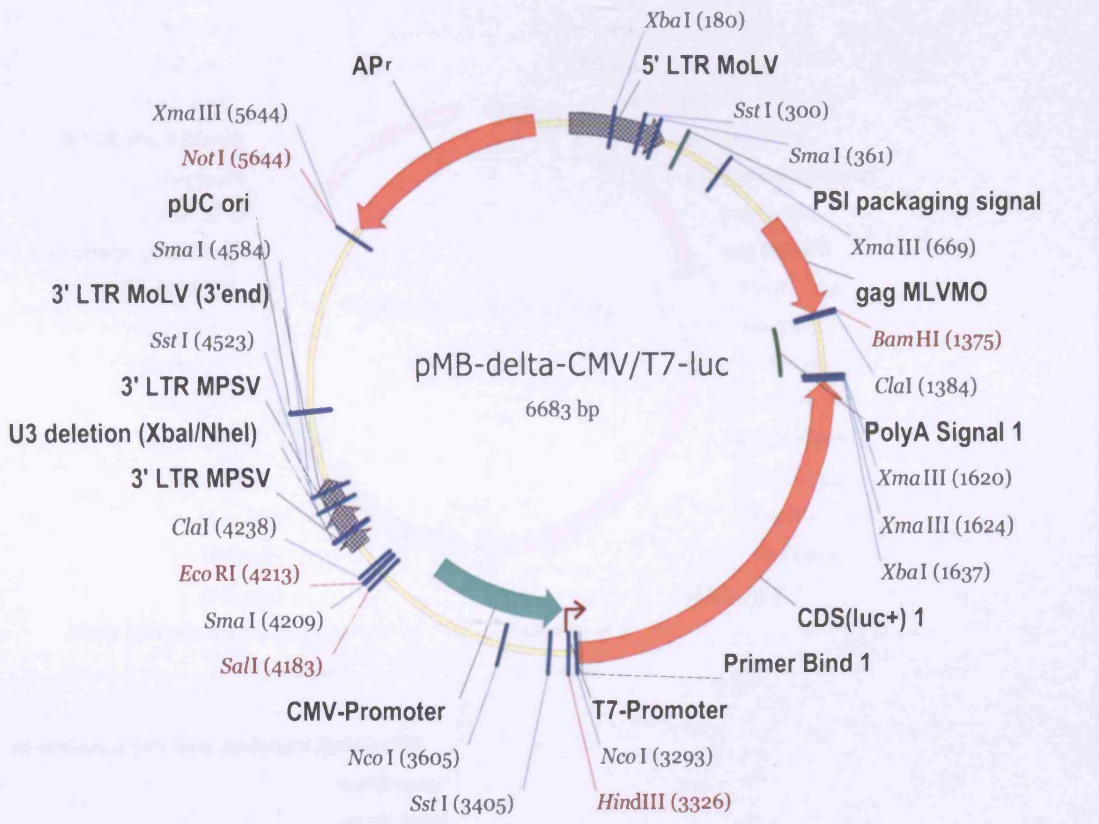
Insert plasmid: pGL3-CMV/T7-luc

Digest: BglII/BamHI

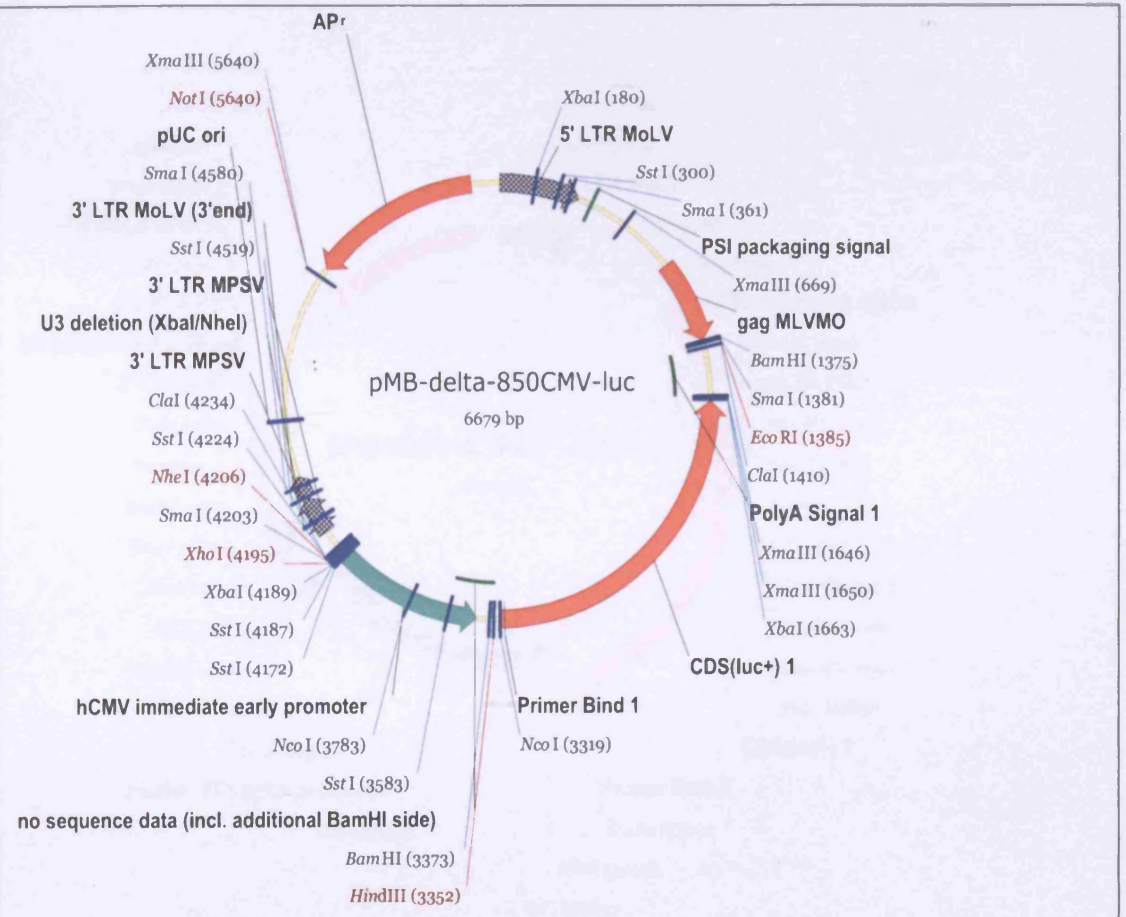
Insert orientation confirmation

Digest: XbaI

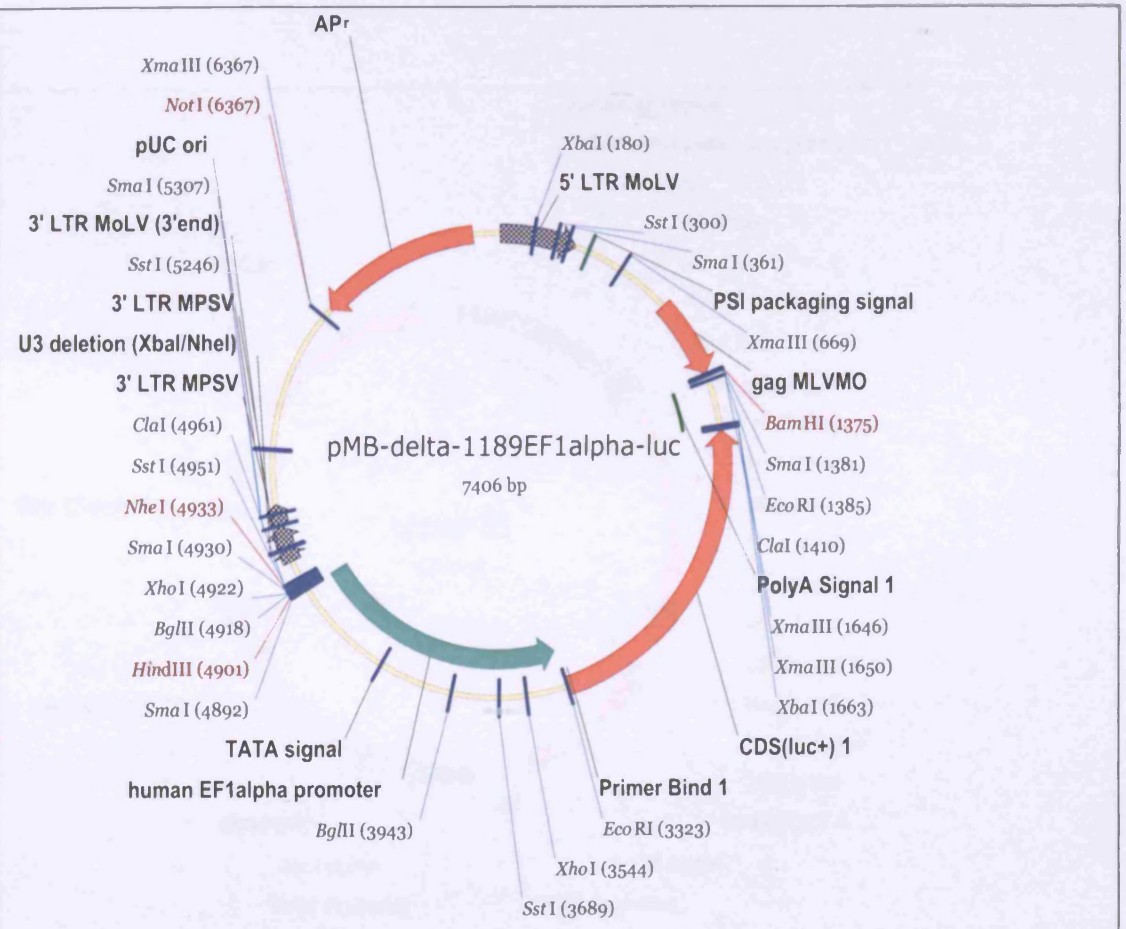
Reference: this thesis



8.3.9 pMBΔ-850CMV-luc

Endogenous Promoter: 5'LTR Exogenous Promoter: CMV850 Modification: enhancer deleted 3'LTR Reporter gene: luciferase Cloning strategy: Backbone plasmid: pMBΔ Digest: ClaI Insert plasmid: pGL3-850CMV-luc Digest: ClaI Insert orientation confirmation Digest: XbaI Reference: this thesis	

8.3.10 pMBΔ-1189EF1α-luc

Endogenous Promoter: 5'LTR Exogenous Promoter: EF1α (1189bp) Modification: enhancer deleted 3'LTR Reporter gene: luciferase Cloning strategy: Backbone plasmid: pMBΔ Digest: ClaI Insert plasmid: pGL3-1189EF1α-luc Digest: ClaI Insert orientation confirmation Digest: XbaI Reference: this thesis	

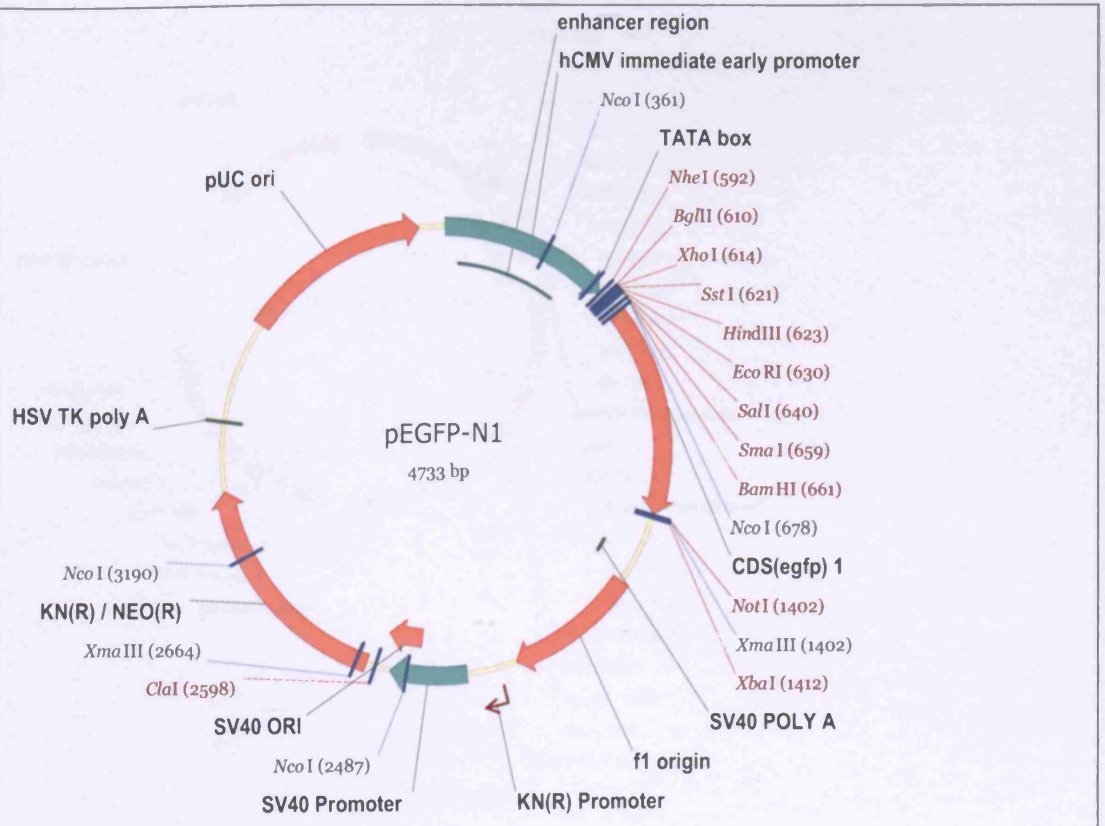
8.4 Plasmids based on pEGFP-N1 and pN1 subclones

8.4.1 pEGFP-N1

Promoter: CMV IE

Reporter gene: eGFP

Reference: Clontech



8.4.2 pN1-CMV-PU.1-eGFP

Promoter: murine PU.1

Reporter gene: eGFP

Cloning strategy:

Backbone plasmid: pEGFP-N1

Digest: BamHI

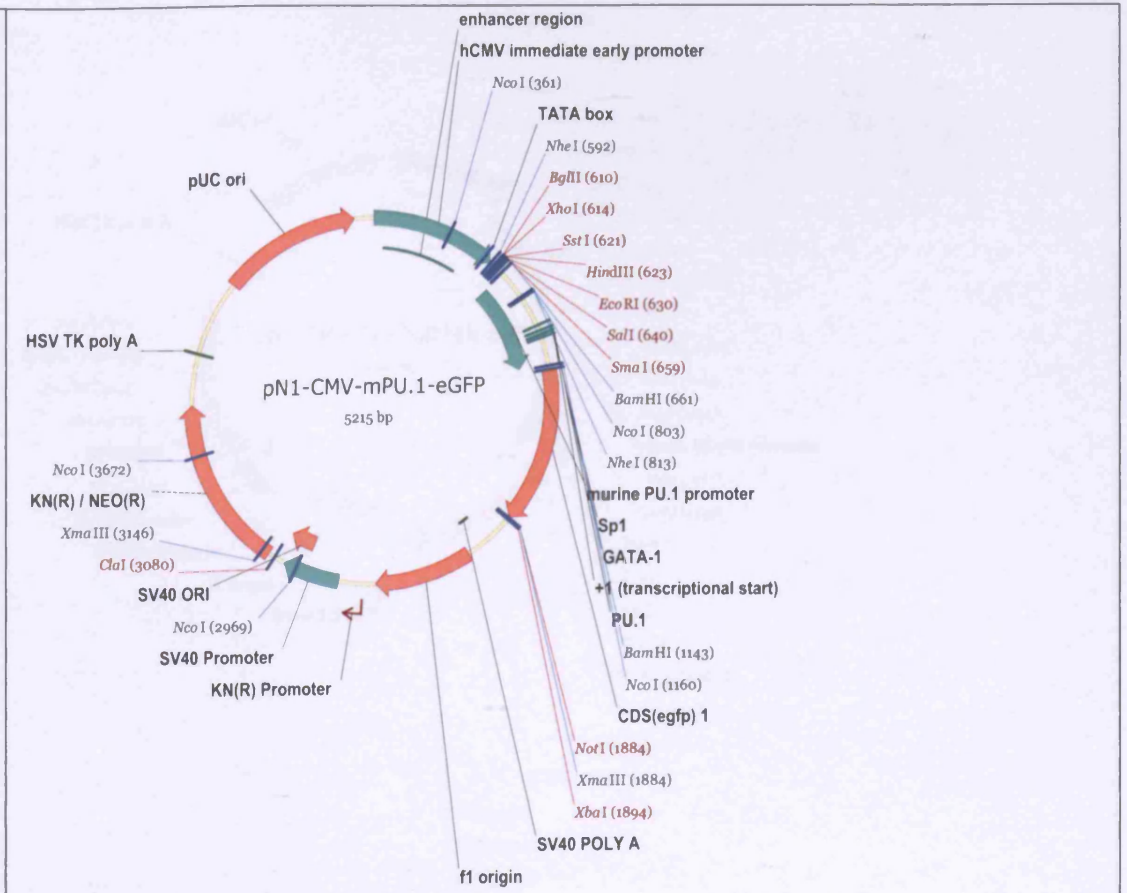
Insert plasmid: pmPU.1-334/luc

Digest: BamHI

Insert orientation confirmation

Digest: NheI (221bp correct / 399bp wrong orientation)

Reference: this thesis



8.4.3 pN1-CMV-1672CD11b-eGFP

Promoter: CD11b (1672bp)

Reporter gene: eGFP

Cloning strategy:

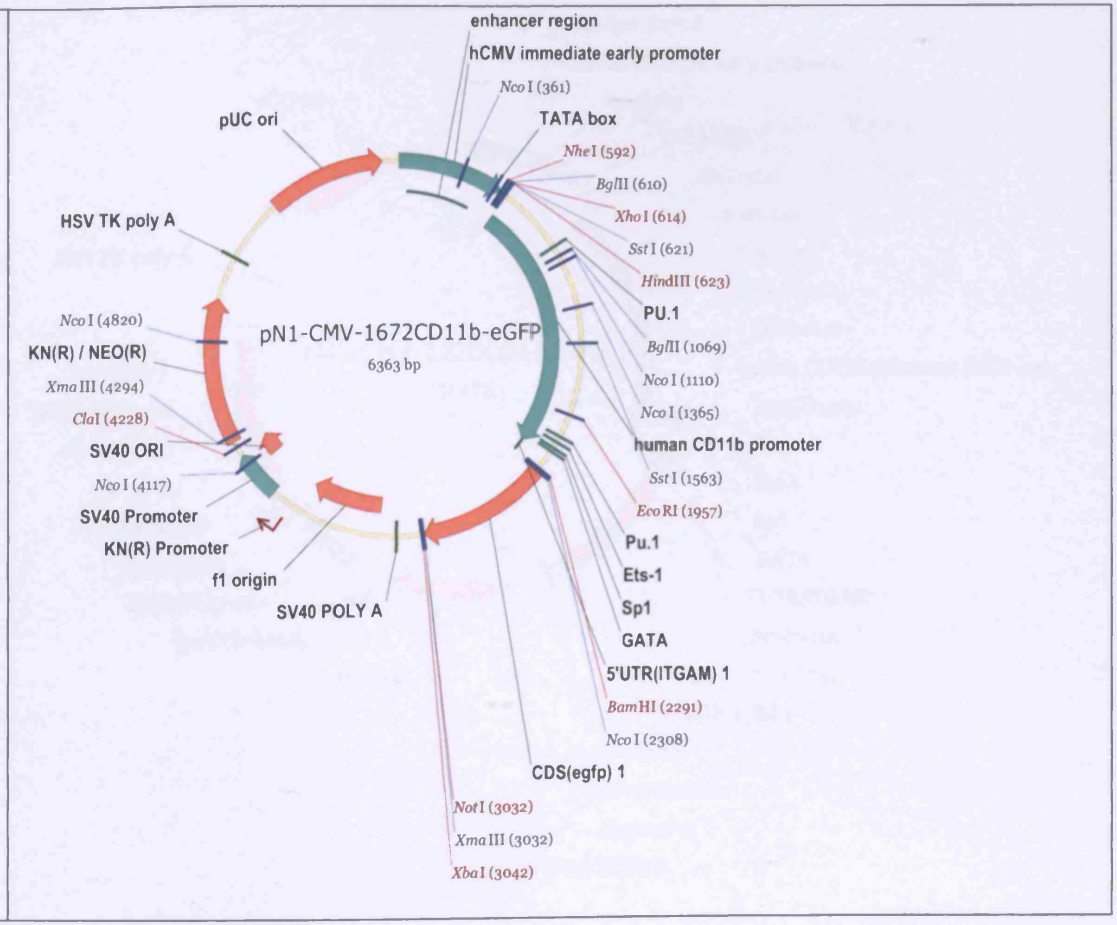
Backbone plasmid: pEGFP-N1

Digest: HindIII/BamHI

Insert plasmid: pΦGH-CD11b

Digest: HindIII/BamHI

Reference: this thesis



8.4.4 pN1-CMV-1227CD11b-eGFP

Promoter: CD11b (1227bp)

Reporter gene: eGFP

Cloning strategy:

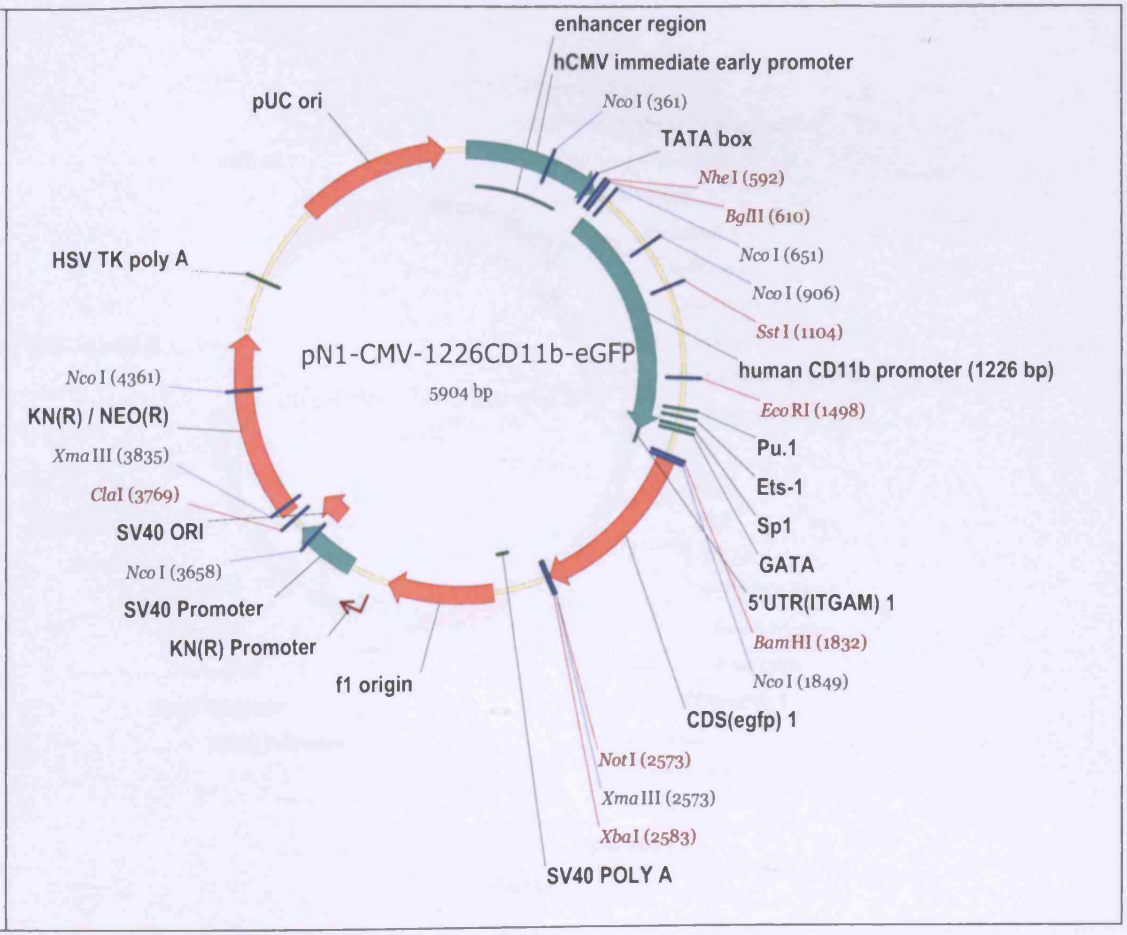
Backbone plasmid: pN1-CMV-1672CD11b-eGFP

Digest: BglII

Deletion: 464bp

Religation

Reference: this thesis



8.4.5 pN1-CMV-732CD11b-eGFP

Promoter: CD11b (732bp)

Reporter gene: eGFP

Cloning strategy:

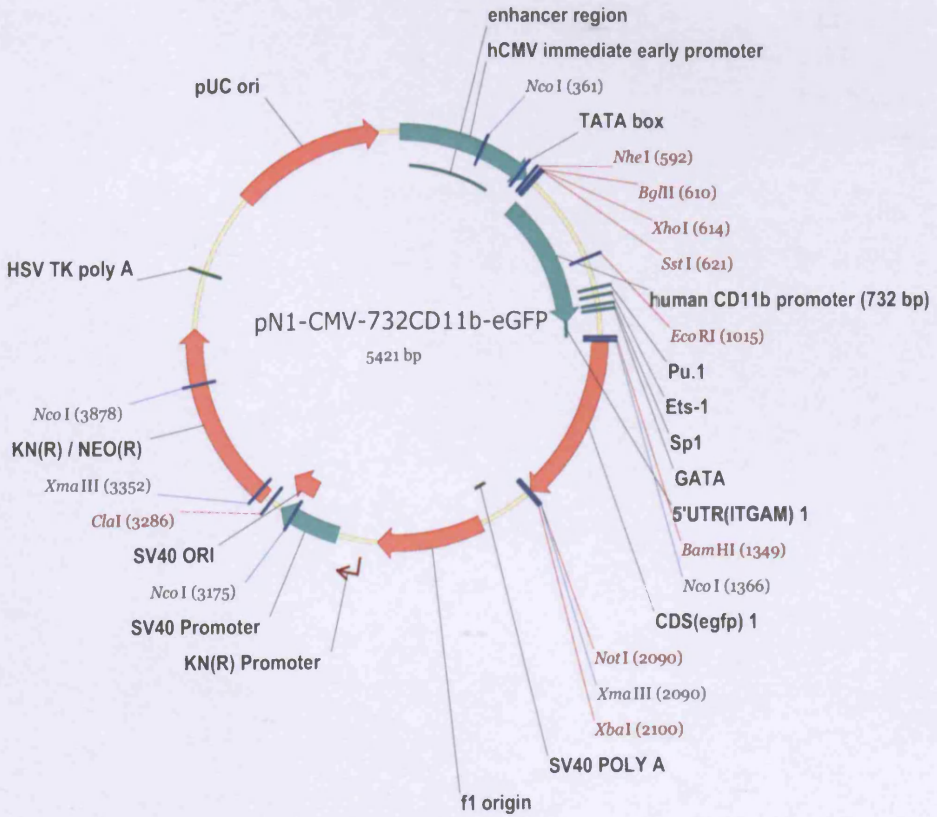
Backbone plasmid: pN1-CMV-1672CD11b-eGFP

Digest: SstI

Deletion: 982bp

Religation

Reference: this thesis



8.4.6 pN1-CMV-1672CD11b-luc

Promoter: CD11b (1672bp)

Reporter gene: luciferase

Cloning strategy:

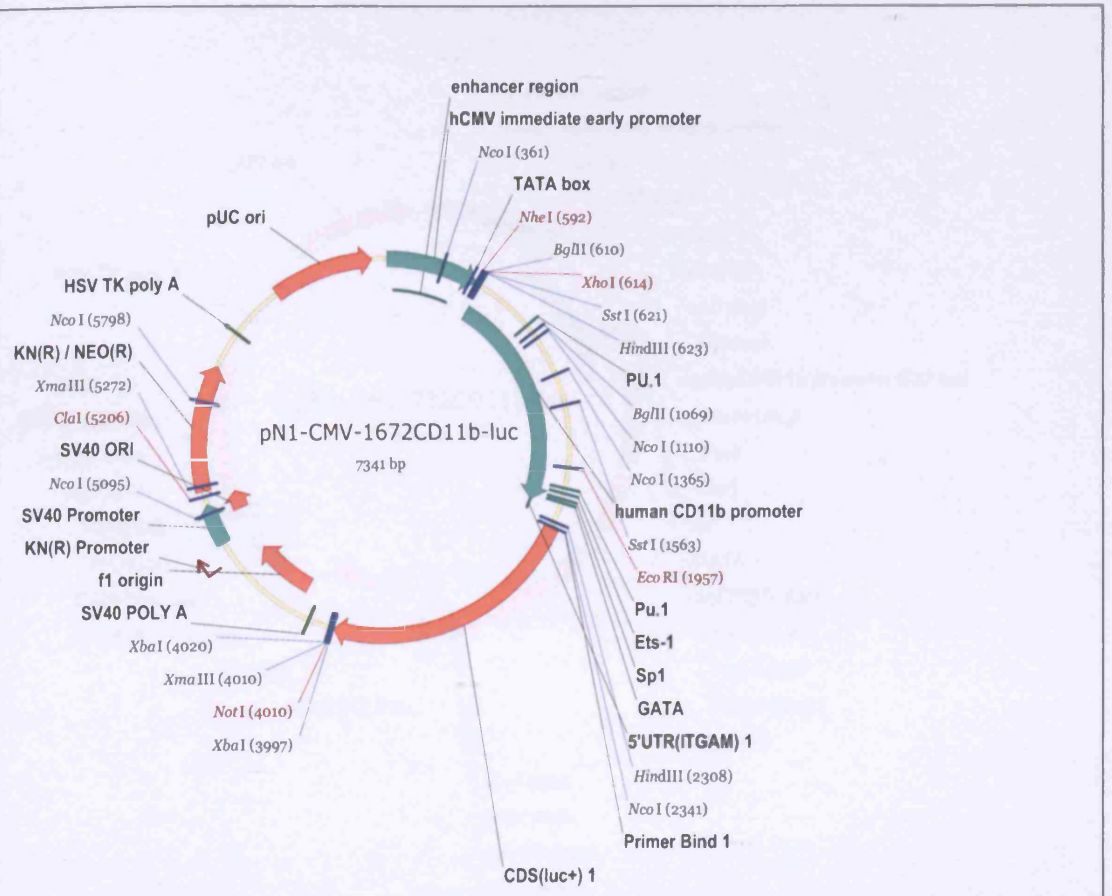
Backbone plasmid: pN1-CMV-1672CD11b-eGFP

Digest: HindIII/NotI

Insert plasmid: pGL3-Basic

Digest: BglII/XmaIII

Reference: this thesis, (Schwickerath et al., 2004)



8.4.7 pN1-CMV-732CD11b-luc

Promoter: CD11b (732bp)

Reporter gene: luciferase

Cloning strategy:

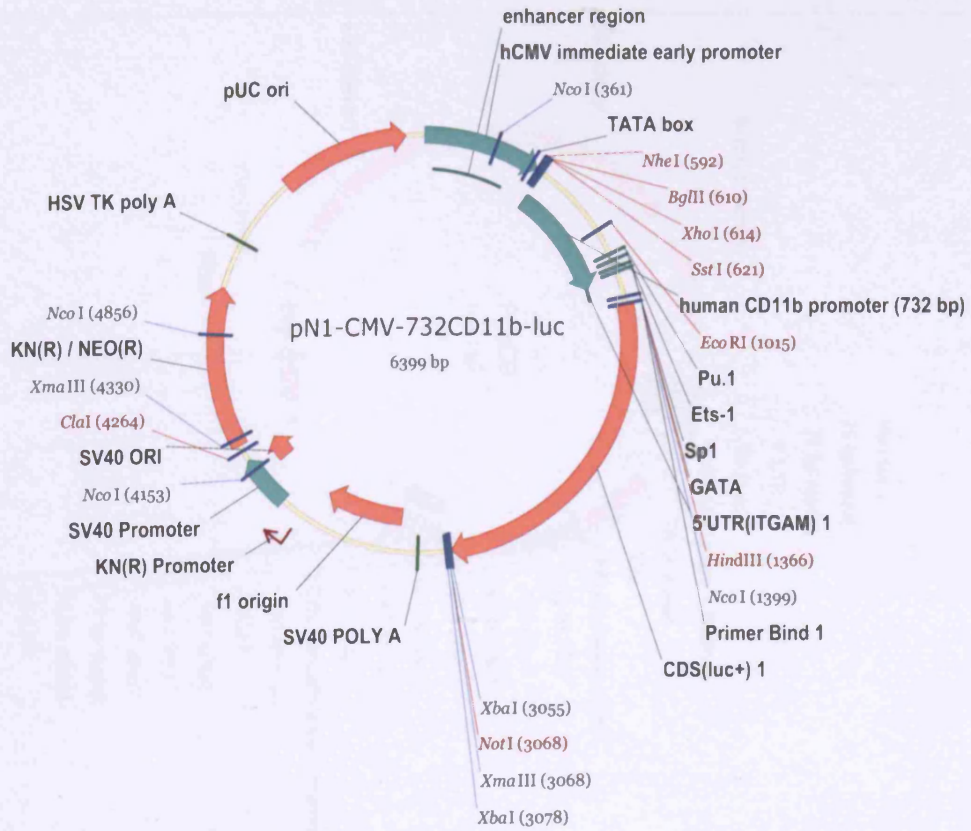
Backbone plasmid: pN1-CMV-1672CD11b-luc

Digest: SstI

Deletion: 982bp

Religation

Reference: this thesis, (Schwickerath et al., 2004)



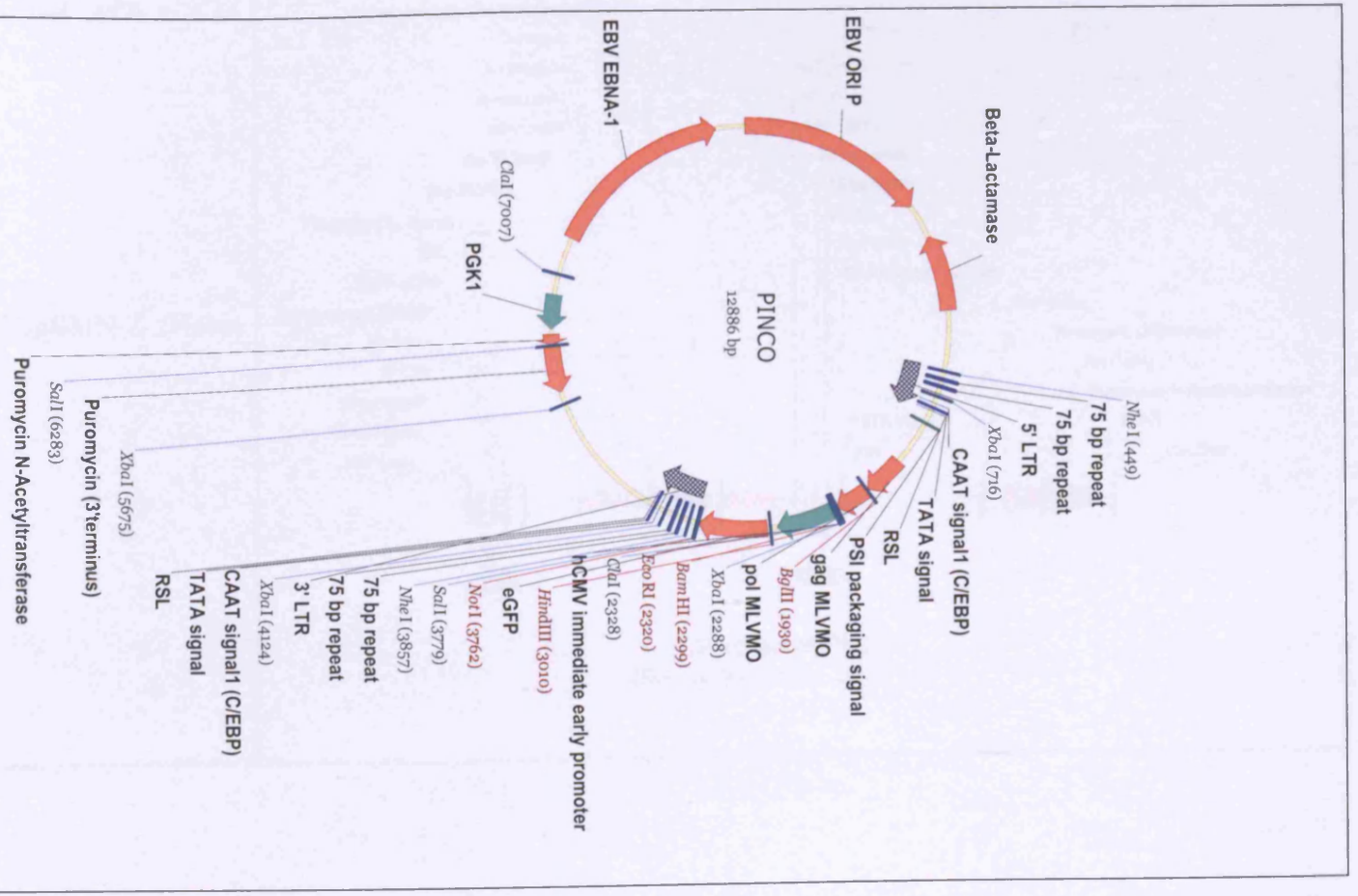
8.5 Retroviral Vectors based on PINCO and relevant subclones

8.5.1 PINCO (equal to PINCO-CMV-eGFP)

A schematic representation of the retroviral vector PINCO (equal to PINCO-CMV-eGFP), presented as circular plasmid (12886bp).

Reference: (Grignani et al., 1998), Plasmid DNA and DNA sequence data were obtained as kind gift from Prof. Dr. Pier Giuseppe Pelicci, Milan, Italy

Additonal positions of restriction sites are indicated, which were left out earlier (Figure 19), but are essential for the cloning strategies applied to the following PINCO derivates.

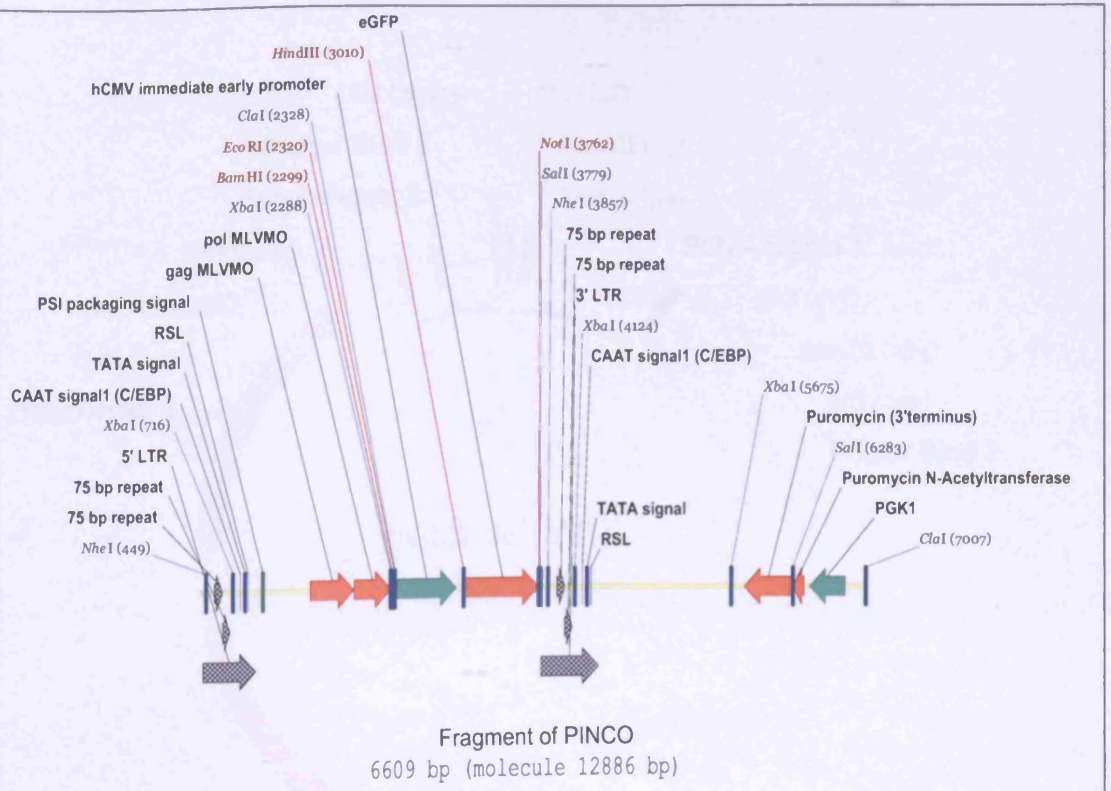


For better visulisation, a linear fragment of PINCO from a few base pairs before the position of *Nhe*I (449) to *Cla*I (7007) is presented here on the right.

Exogenous Promoter: CMV IE

Reporter gene: eGFP

Backbone plasmid (not shown): pLZRSpBMN-Z (Nolan Laboratory, Stanford)



8.5.2 pGL3-ΔNX

Promoter: no promoter

Reporter gene: luciferase

Cloning strategy:

Backbone plasmid: pGL3-Basic

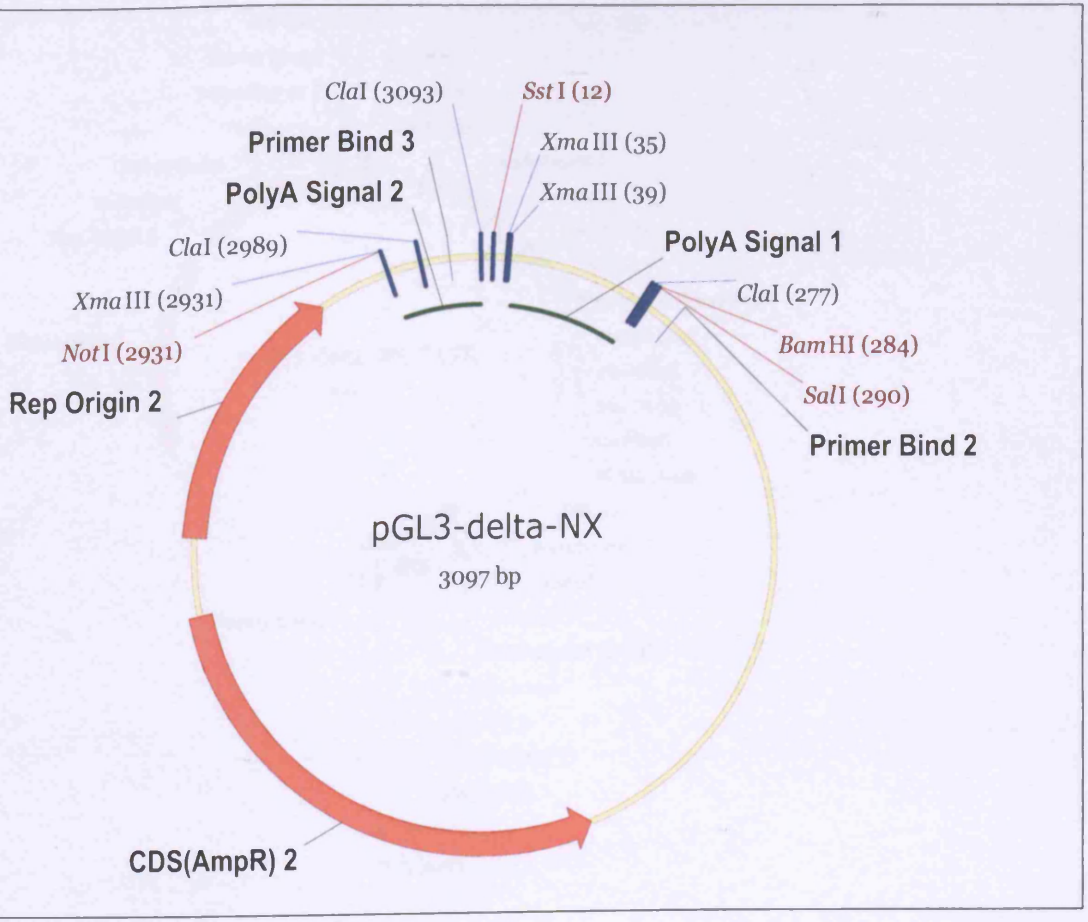
Digest: NheI/XbaI

Deletion: 1721bp

Religation

Reference: this thesis

Equal to pGL3-Δ-NX (Schwickerath et al., 2004)



8.5.3 pGL3-ΔNX-3'LTR

Insert: 3'LTR

Cloning strategy:

Backbone plasmid: pGL3-ΔNX

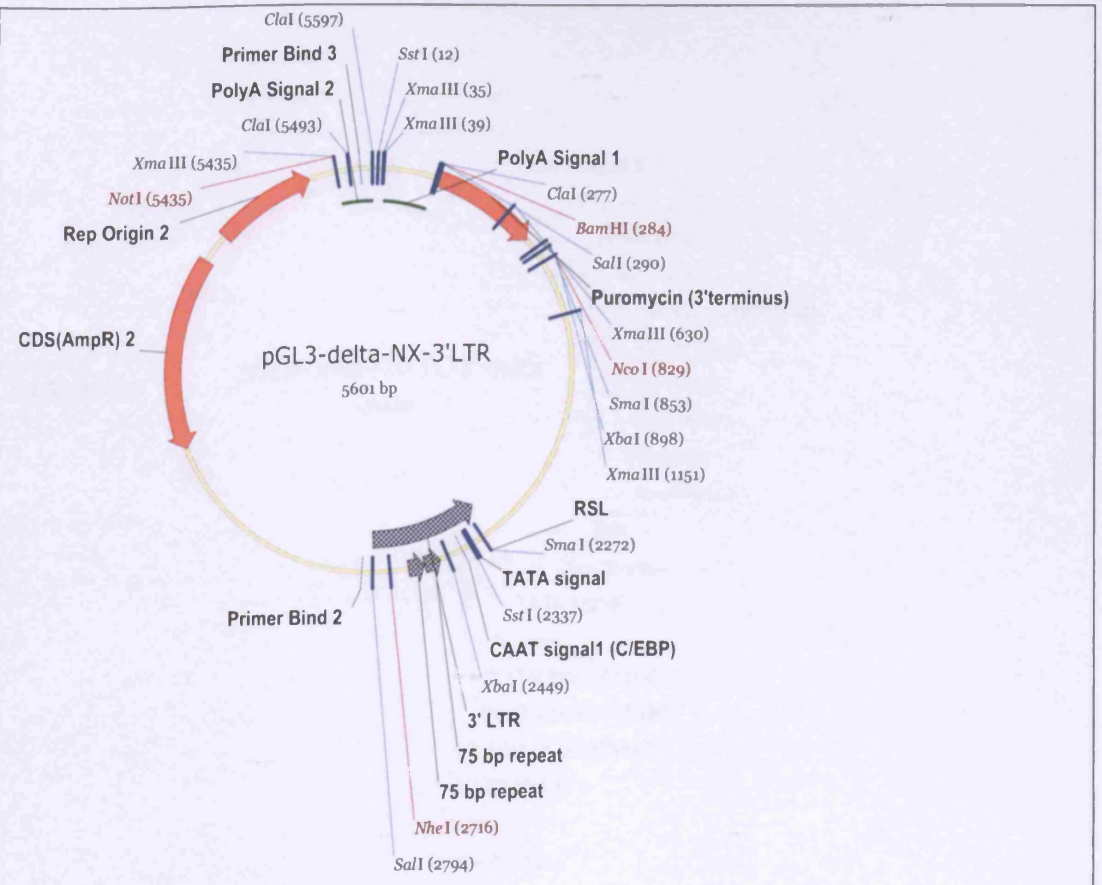
Digest: SalI

Insert plasmid: PINCO

Digest: SalI

Reference: this thesis

Equal to pGL3-ΔNX-LTR (Schwickerath et al., 2004)



8.5.4 pGL3-ΔNX-3'LTRΔ

Insert: enhancer deleted 3'LTR

Cloning strategy:

Backbone plasmid: pGL3-ΔNX-3'LTR

Digest: NheI / XbaI

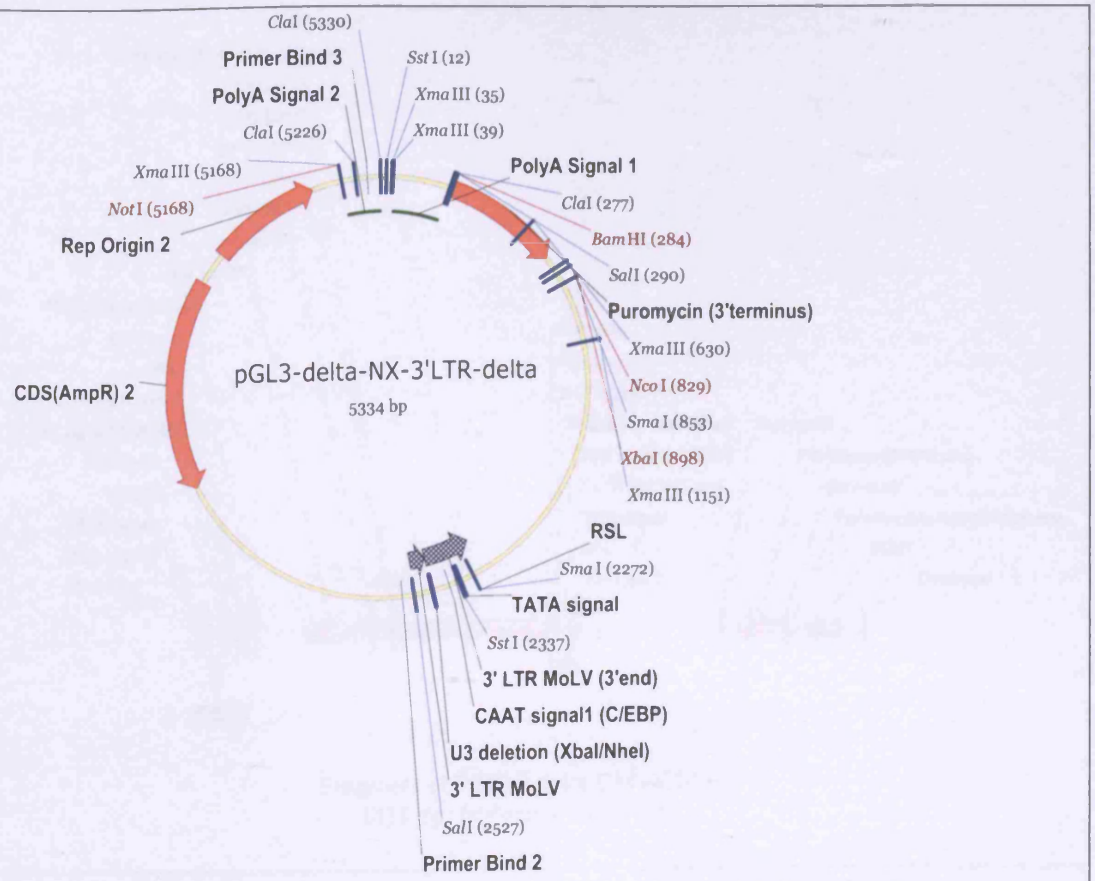
Deletion: 267bp

Ligation of XbaI/XbaI Fragment to backbone plasmid

Orientation test:

Reference: this thesis

Equal to pGL3-ΔNX-ΔLTR (Schwickerath et al., 2004)



8.5.5 PINCOΔ (equal to PINCOΔ-CMV-eGFP)

(Equal to PINCOΔ-CMV-eGFP)

Exogenous Promoter: CMV IE

Reporter gene: eGFP

Modification: enhancer deleted 3'LTR

Cloning strategy:

Backbone plasmid: PINCO

Digest: SalI

Insert plasmid: pGL3-ΔNX-3'LTRΔ

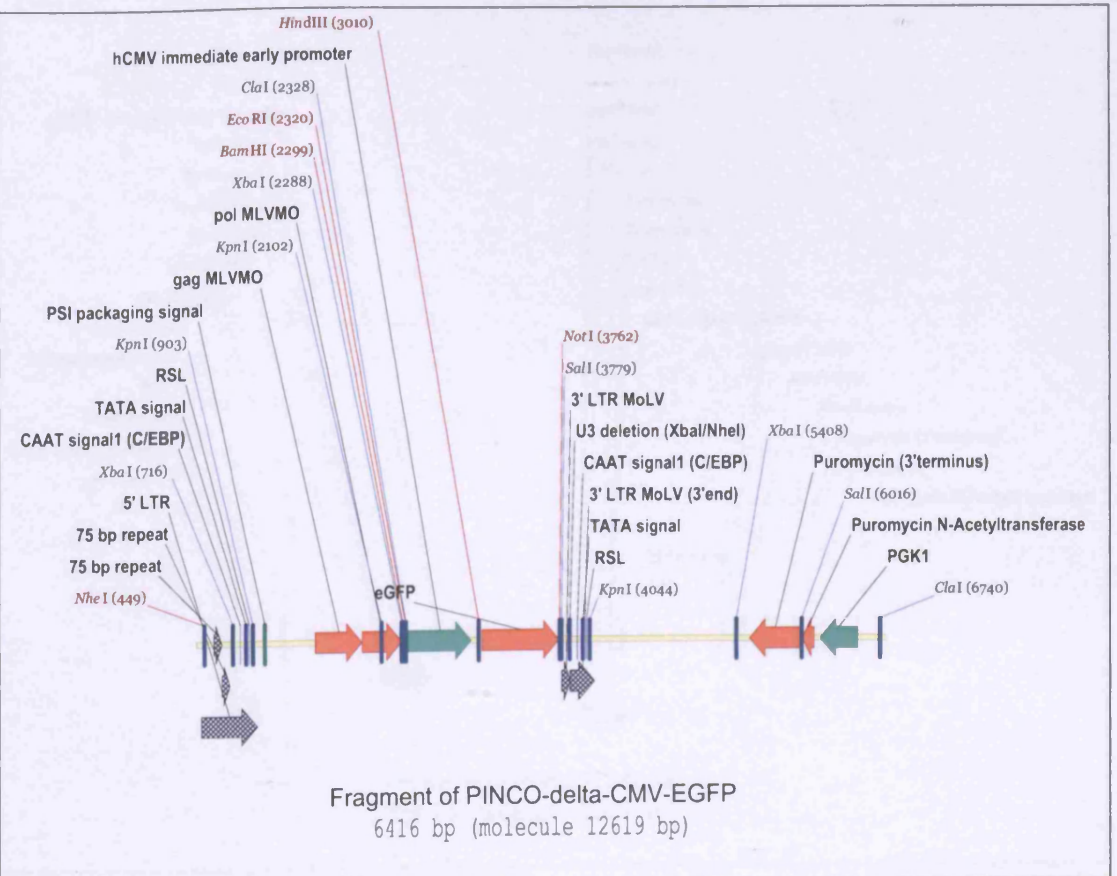
Digest: SalI

Insert orientation confirmation

Digest: KpnI

Reference: this thesis

Equal to PINCOΔ (Schwickerath et al., 2004)



8.5.6 PINCO-CMV-luc

Exogenous Promoter: CMV IE

Reporter gene: luciferase

Cloning strategy:

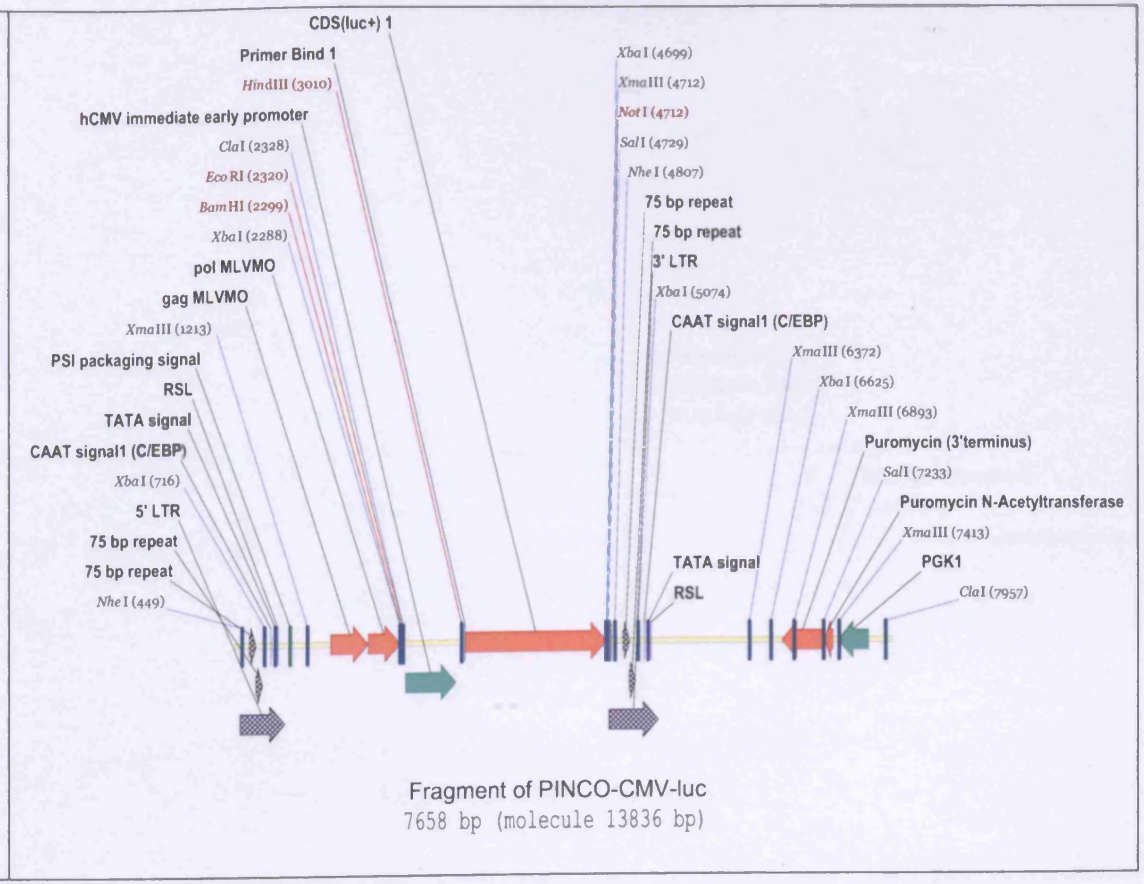
Backbone plasmid: PINCO

Digest: HindIII/NotI

Insert plasmid: pGL3-Basic

Digest: HindIII/XmaIII

Reference: this thesis



8.5.7 PINCOΔ-CMV-luc

Exogenous Promoter: CMV

Reporter gene: luciferase

Cloning strategy:

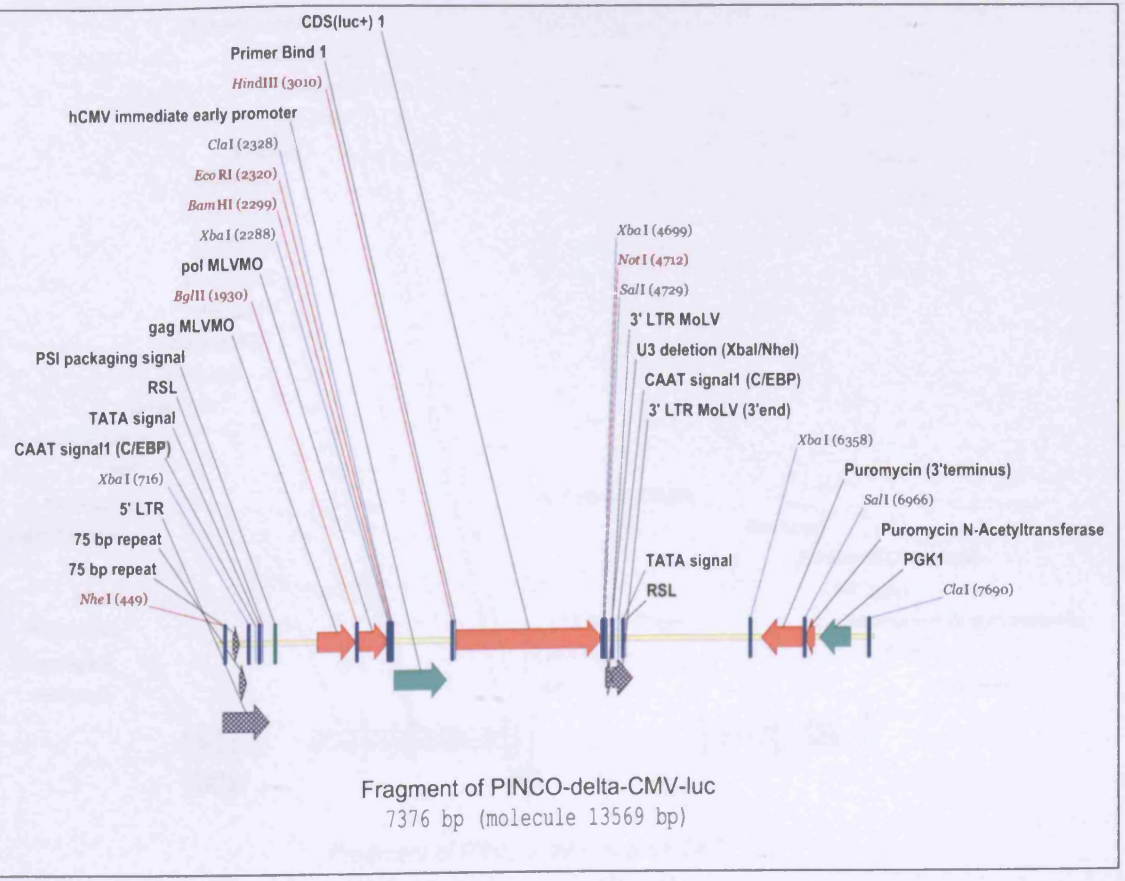
Backbone plasmid: PINCOΔ

Digest: HindIII/NotI

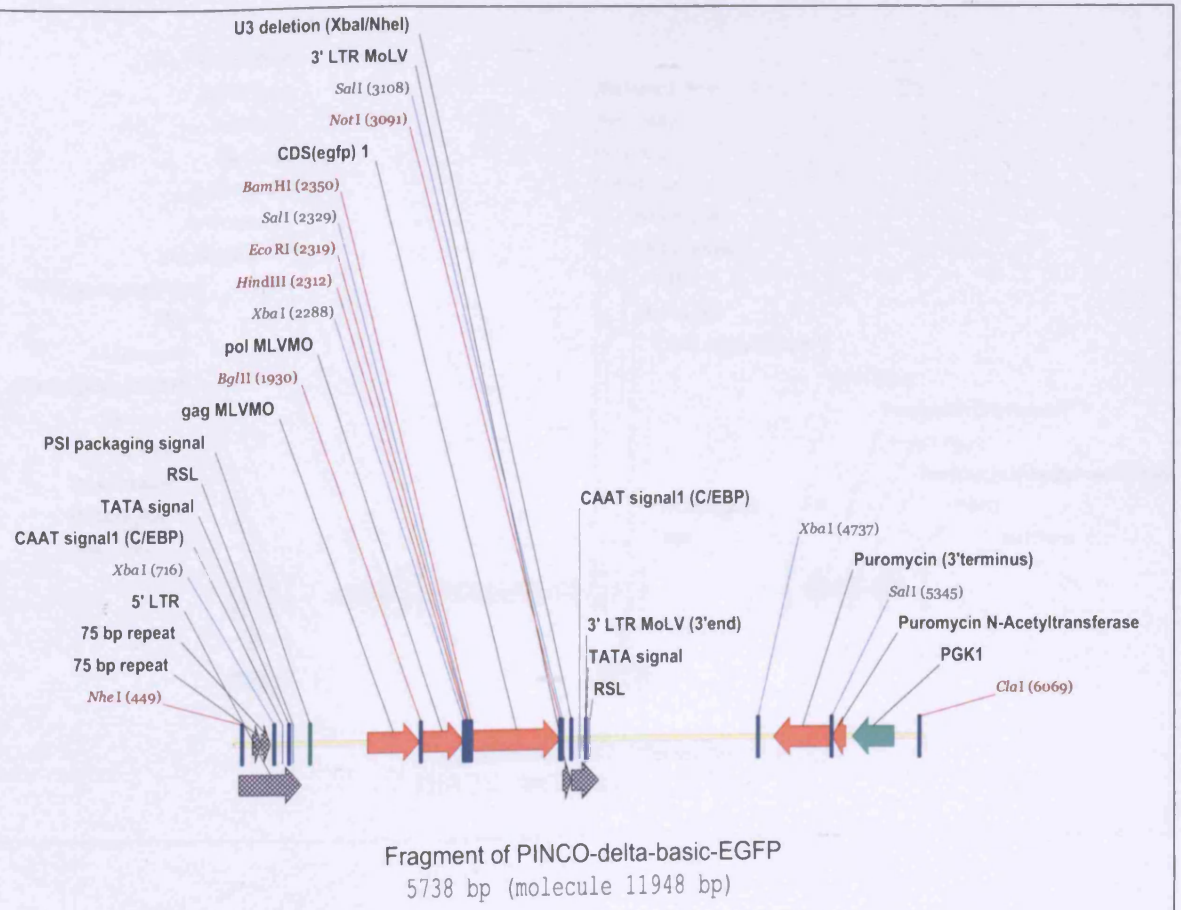
Insert plasmid: pGL3-Basic

Digest: HindIII/XmaIII

Reference: this thesis



8.5.8 PINCOΔ-Basic-eGFP

Endogenous Promoter: 5'LTR Exogenous Promoter: no promoter Reporter gene: eGFP Modification: CMV deletion / enhancer deleted 3'LTR Cloning strategy: Backbone plasmid: PINCOΔ Digest: BamHI/NotI Insert plasmid: pEGFP-N1 Digest: BglII/NotI Insert orientation confirmation Digest: SalI Reference: this thesis Equal to Δ-LTR(eGFP) (Schwickerath et al., 2004)	

8.5.9 PINCO-Basic-luc

Endogenous Promoter: 5'LTR

Exogenous Promoter: no

Reporter gene: luciferase

Modification: CMV deletion

Cloning strategy:

Backbone plasmid:

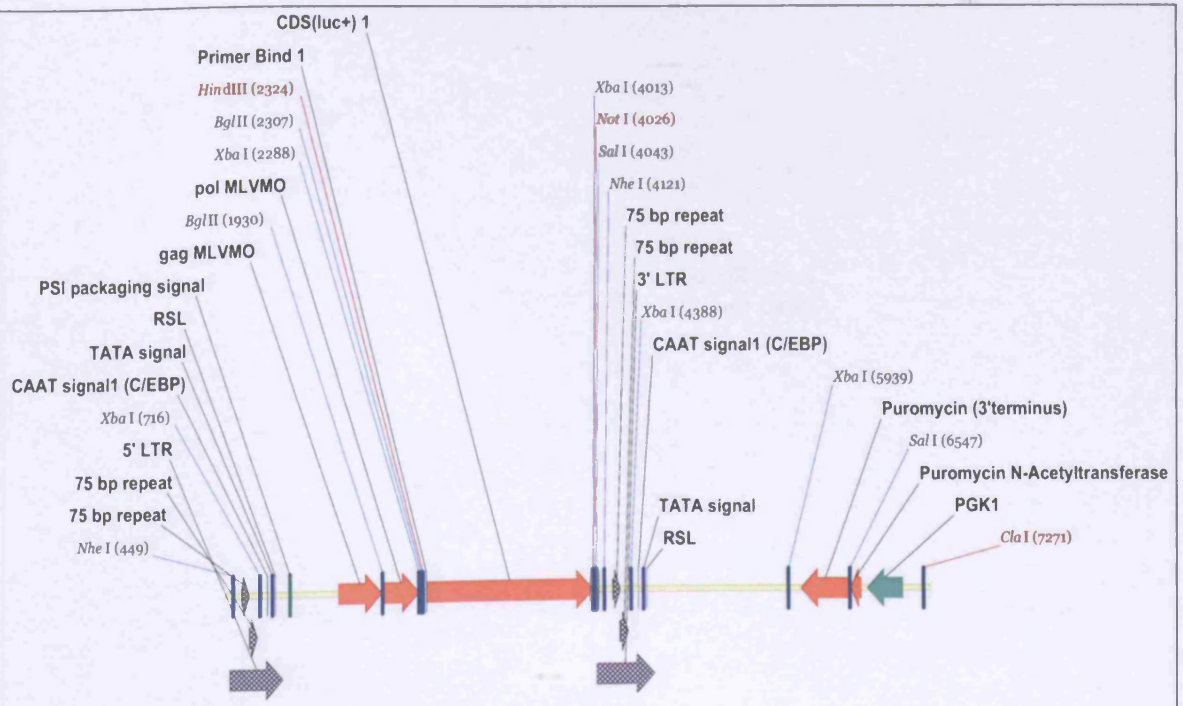
PINCO-Basic-eGFP

Digest: XhoI/NotI

Insert plasmid: pGL3-Basic

Digest: XhoI/XmaIII

Reference: this thesis



8.5.10 PINCOΔ-Basic-luc

Endogenous Promoter: 5'LTR

Exogenous Promoter: no

Reporter gene: luciferase

Modification: CMV deletion / enhancer deleted 3'LTR

Cloning strategy:

Backbone plasmid:

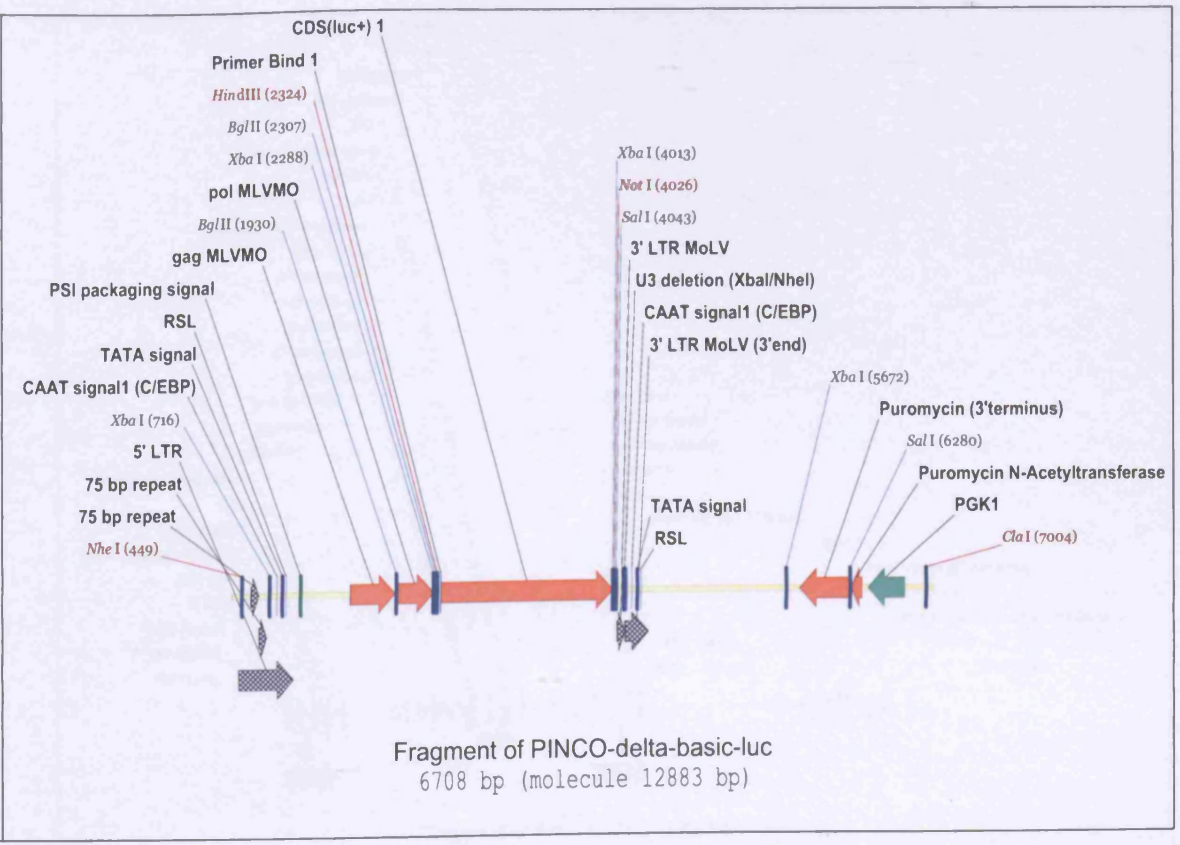
PINCOΔ- Basic-eGFP

Digest: XhoI/NotI

Insert plasmid: pGL3-Basic

Digest: XhoI/XmaIII

Reference: this thesis



8.5.11 PINCO-PU.1-eGFP

Endogenous Promoter: 5'LTR

Exogenous Promoter: PU.1

Reporter gene: eGFP

Cloning strategy:

Backbone plasmid: PINCO (PINCO-CMV-eGFP

Digest: BamHI/NotI

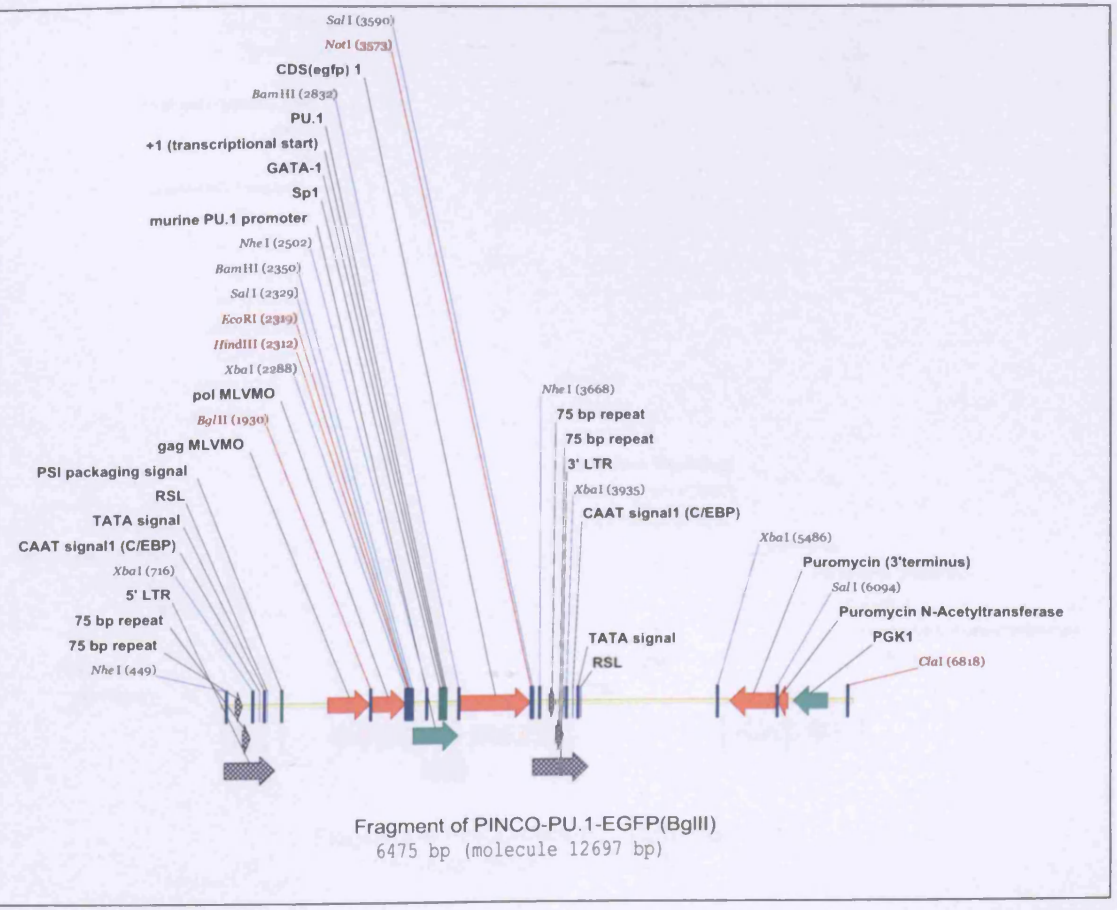
Insert plasmid: pN1-CMV-PU.1-eGFP

Digest: BglII/NotI

Insert orientation confirmation

Digest: NheI

Reference: this thesis



8.5.12 PINCOΔ-PU.1-eGFP

Endogenous Promoter: 5'LTR

Exogenous Promoter: PU.1

Modification: enhancer deleted 3'LTR

Reporter gene: eGFP

Cloning strategy:

Backbone plasmid: PINCOΔ (PINCOΔ-CMV-eGFP)

Digest: BamHI/NotI

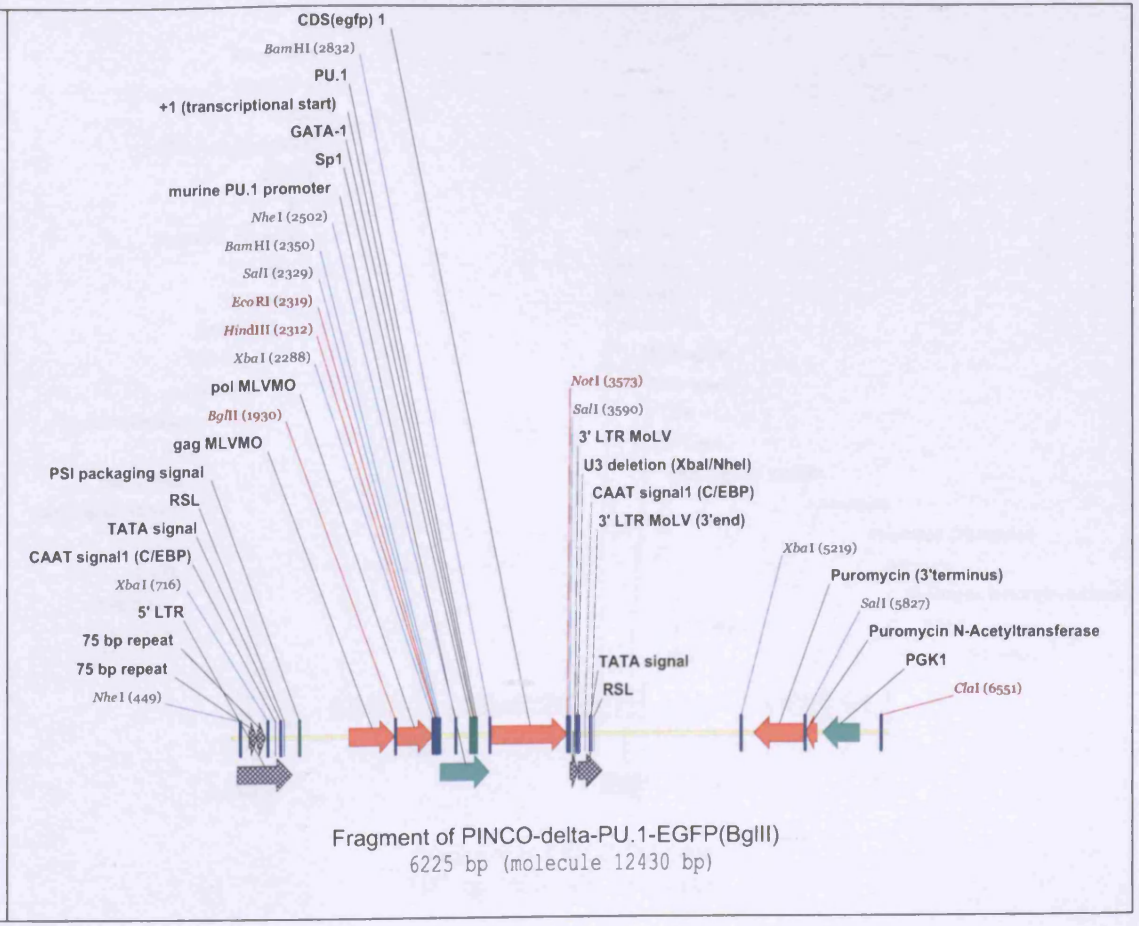
Insert plasmid: pN1-CMV-PU.1-eGFP

Digest: BglII/NotI

Insert orientation confirmation

Digest: NheI

Reference: this thesis



8.5.13 PINCO-PU.1-luc

Endogenous Promoter: 5'LTR

Exogenous Promoter: PU.1

Reporter gene: luciferase

Cloning strategy:

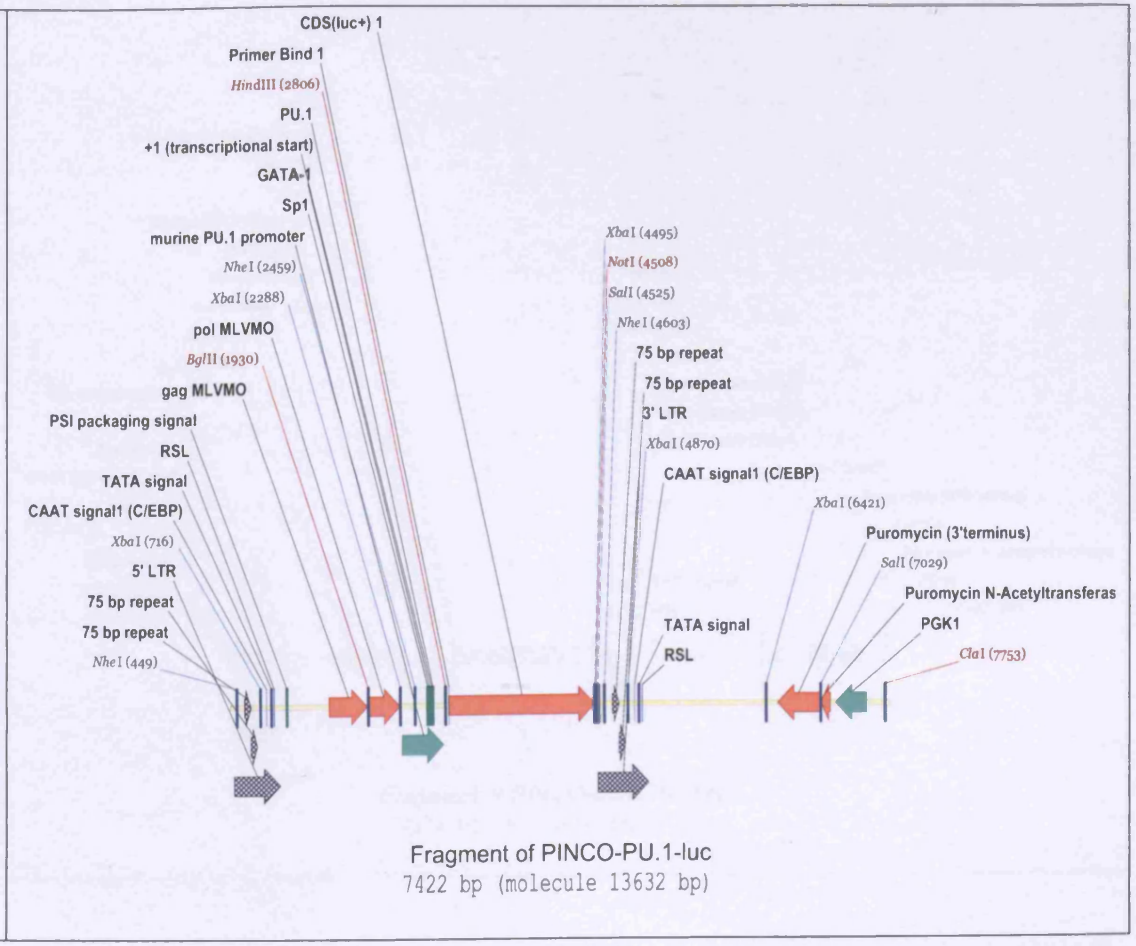
Backbone plasmid: PINCO-1672CD11b-luc)

Digest: XhoI/HindIII

Insert plasmid: pGL3-PU.1-luc

Digest: XhoI/HindIII

Reference: this thesis



8.5.14 PINCOΔ-PU.1-luc

Endogenous Promoter: 5'LTR

Exogenous Promoter: PU.1

Reporter gene: luciferase

Cloning strategy:

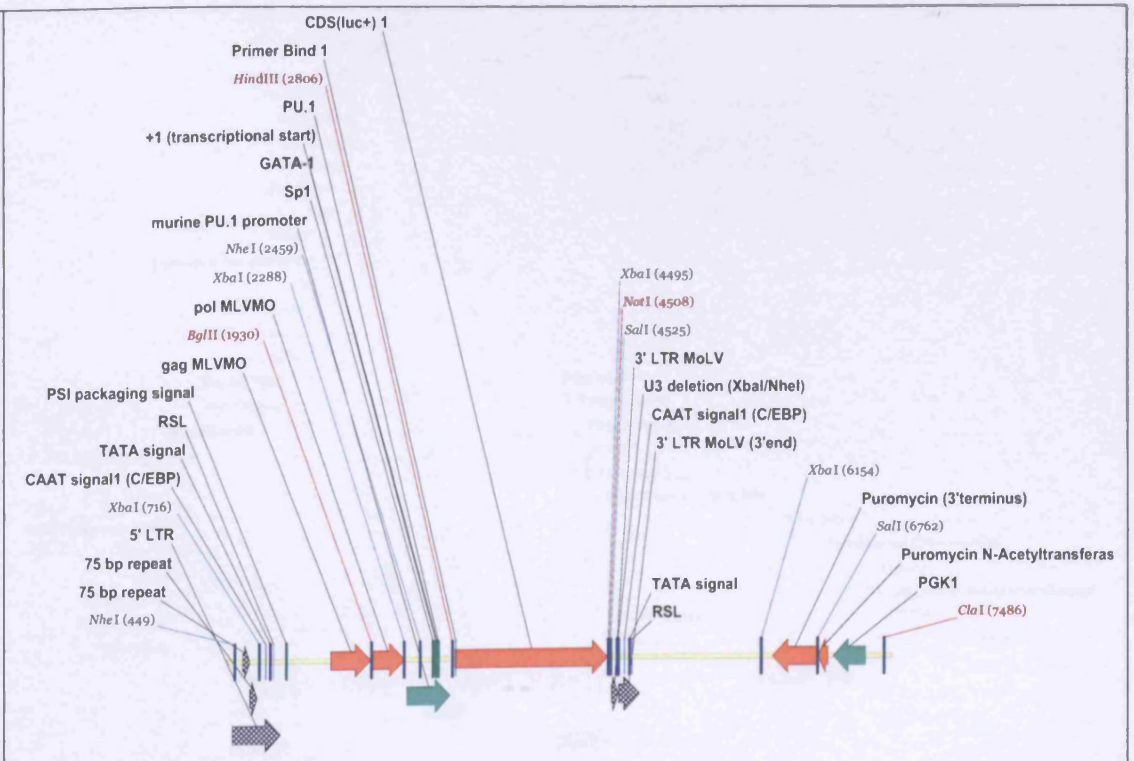
Backbone plasmid: PINCOΔ-1672CD11b-luc

Digest: XhoI/HindIII

Insert plasmid: pGL3-PU.1-luc

Digest: XhoI/HindIII

Reference: this thesis



8.5.15 PINCO-446cfes-eGFP

Endogenous Promoter: 5'LTR

Exogenous Promoter: cfes (446bp)

Reporter gene: eGFP

Cloning strategy:

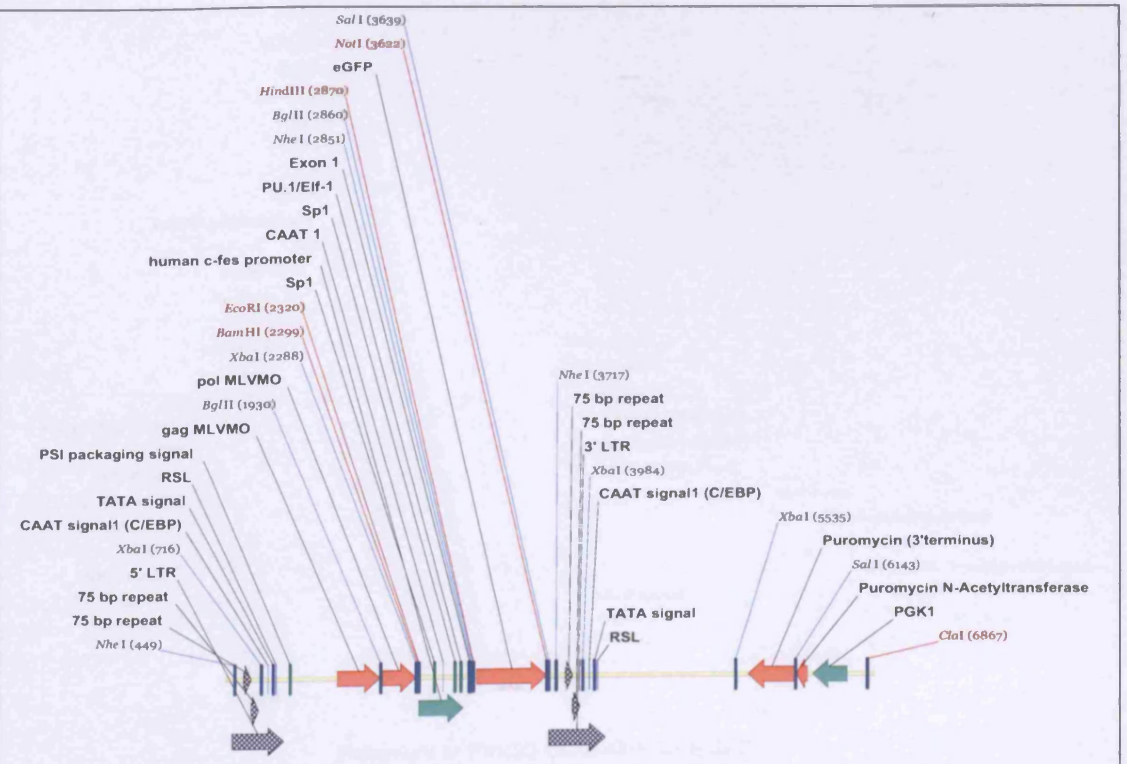
Backbone plasmid: PINCO (PINCO-CMV-eGFP

Digest: HindIII/EcoRI

Insert plasmid: p446

Digest: Tsp509I/HindIII

Reference: this thesis



8.5.16 PINCOΔ-446cfes-eGFP

Endogenous Promoter: 5'LTR

Exogenous Promoter: cfes (446bp)

Modification: enhancer deleted 3'LTR

Reporter gene: eGFP

Cloning strategy:

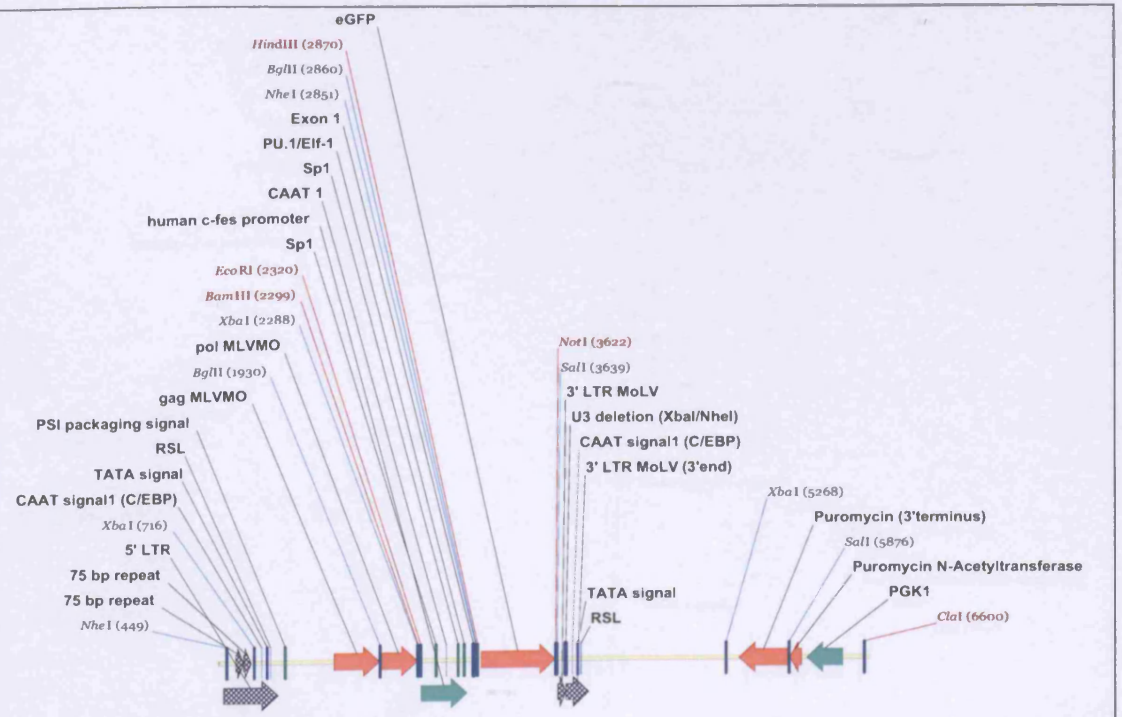
Backbone plasmid: PINCOΔ (PINCOΔ-CMV-eGFP)

Digest: HindIII/EcoRI

Insert plasmid: p446

Digest: Tsp509I/HindIII

Reference: this thesis



8.5.17 PINCO-446cfes-luc

Endogenous Promoter: 5'LTR

Exogenous Promoter: cfes (446bp)

Reporter gene: luciferase

Cloning strategy:

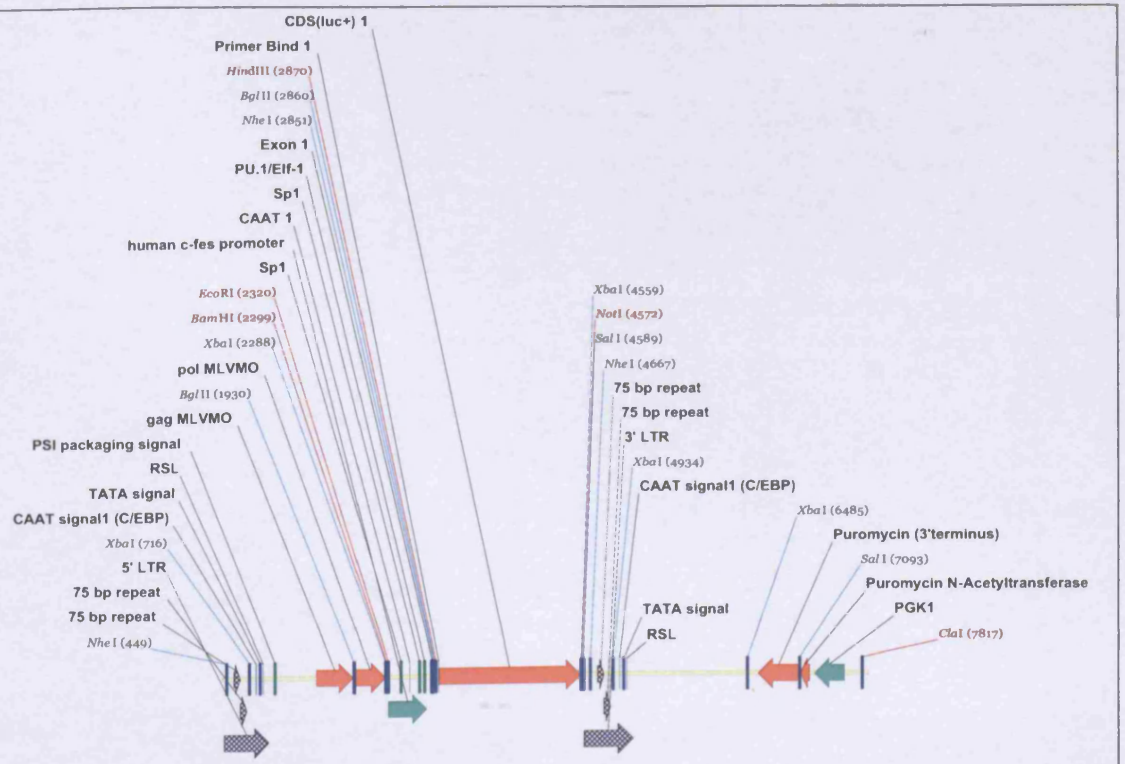
Backbone plasmid: PINCO-CMV-luc

Digest: HindIII/EcoRI

Insert plasmid: p446

Digest: Tsp509I/HindIII

Reference: this thesis



8.5.18 PINCOΔ-446cfes-luc

Endogenous Promoter: 5'LTR

Exogenous Promoter: cfes (446bp)

Reporter gene: luciferase

Cloning strategy:

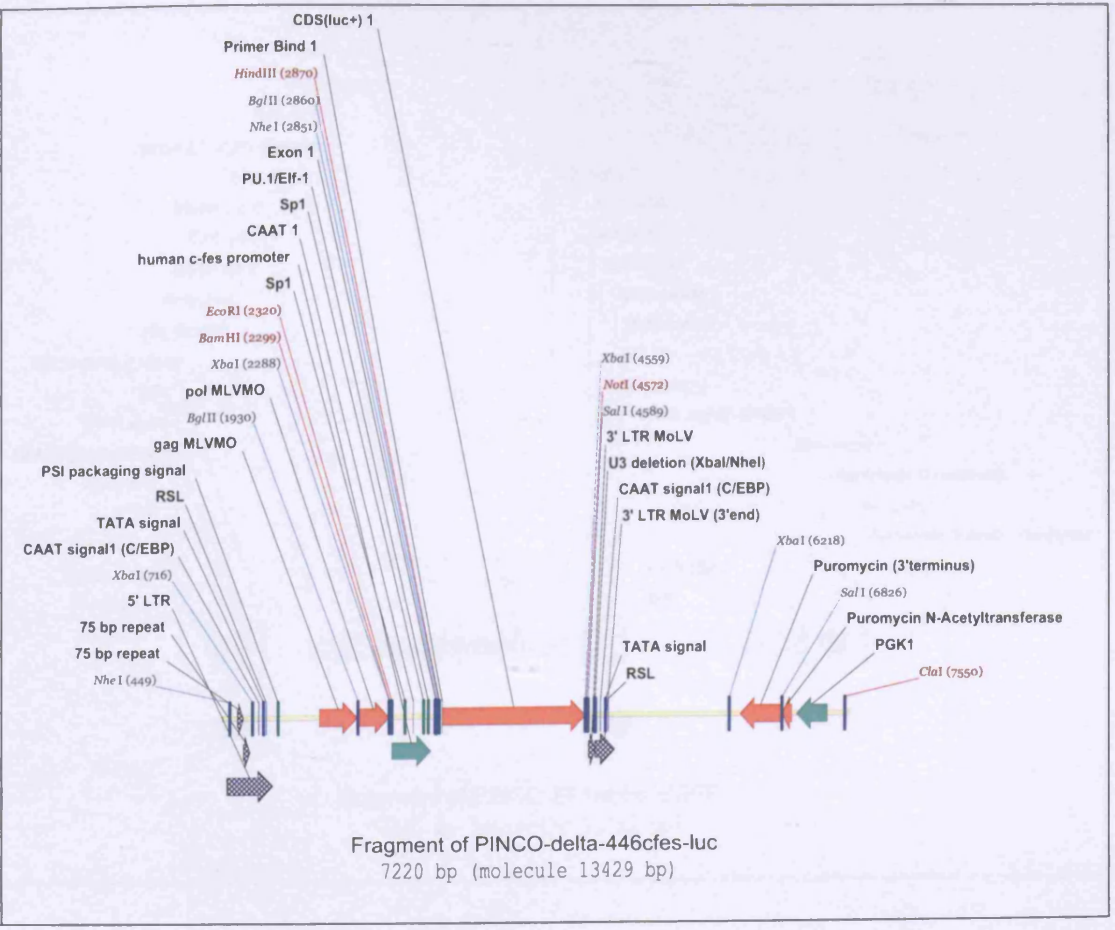
Backbone plasmid: PINCOΔ-CMV-luc

Digest: HindIII/EcoRI

Insert plasmid: p446

Digest: Tsp509I/HindIII

Reference: this thesis



8.5.19 PINCO-1189EF1α-eGFP

Endogenous Promoter: 5'LTR

Exogenous Promoter: EF1α (1189bp)

Reporter gene: eGFP

Cloning strategy:

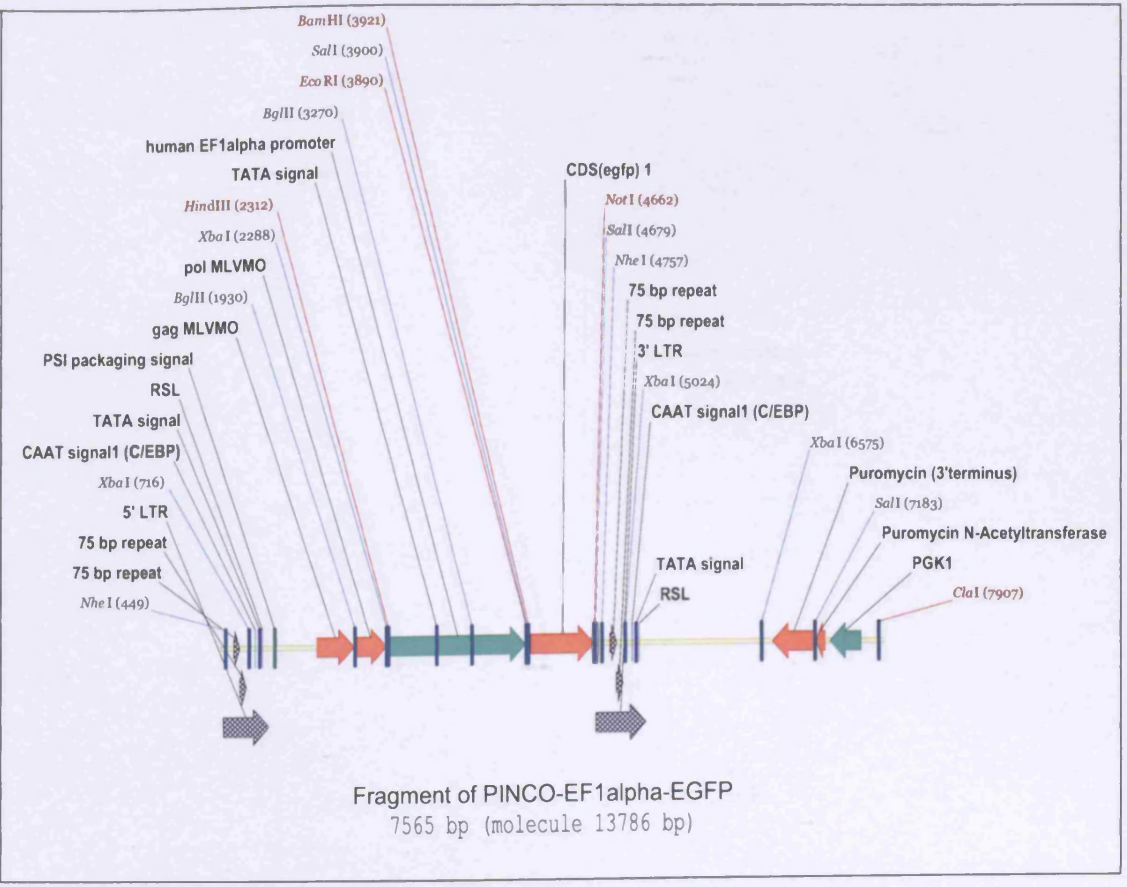
Backbone plasmid: PINCO-eGFP-Basic

Digest: HindIII/NotI

Insert plasmid: pGL3-EF1α-eGFP

Digest: HindIII/NotI

Reference: this thesis



8.5.20 PINCOΔ-1189EF1α-eGFP

Endogenous Promoter: 5'LTR

Exogenous Promoter: EF1α (1189bp)

Reporter gene: eGFP

Cloning strategy:

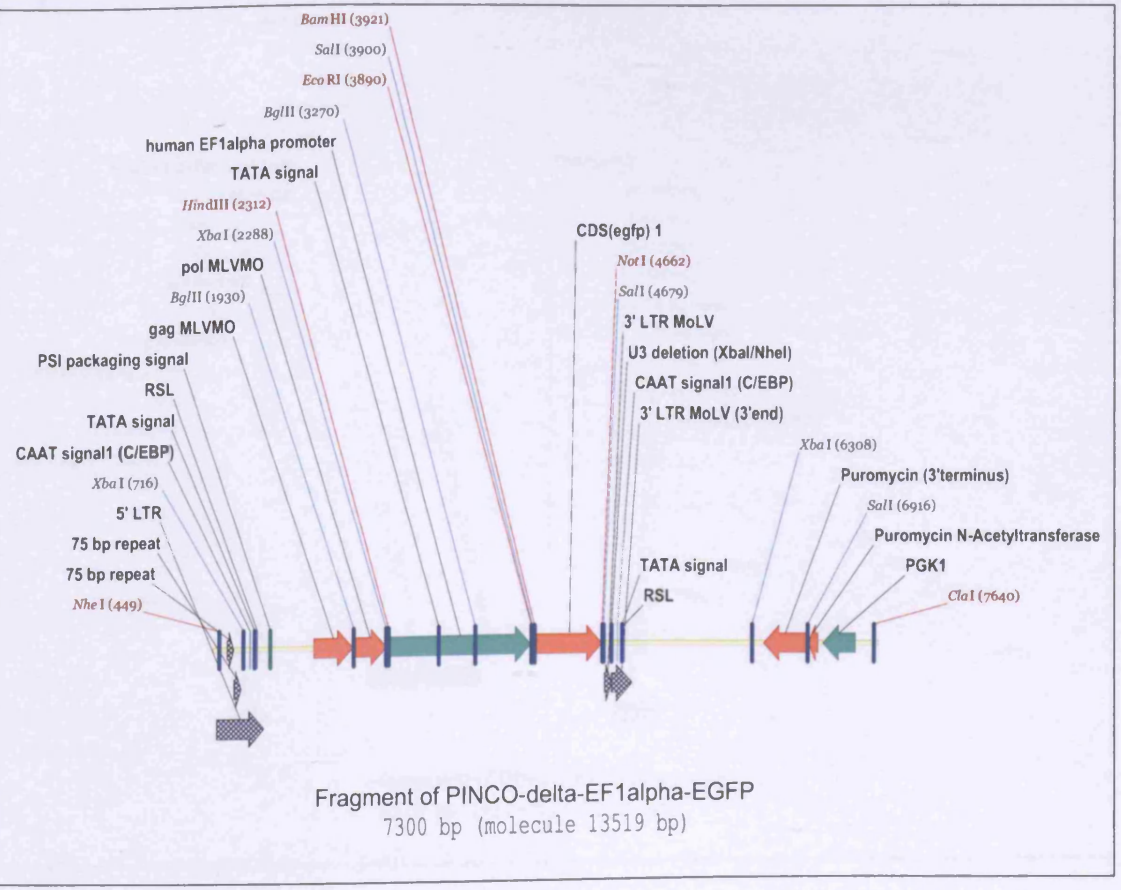
Backbone plasmid: PINCOΔ-eGFP-Basic

Digest: HindIII/NotI

Insert plasmid: pGL3-EF1α-eGFP

Digest: HindIII/NotI

Reference: this thesis



8.5.21 PINCO-1189EF1α-luc

Endogenous Promoter: 5'LTR

Exogenous Promoter: EF1α (1189bp)

Reporter gene: luciferase

Cloning strategy:

Backbone plasmid: PINCO-1189EF1α-eGFP

Digest: BamHI/NotI

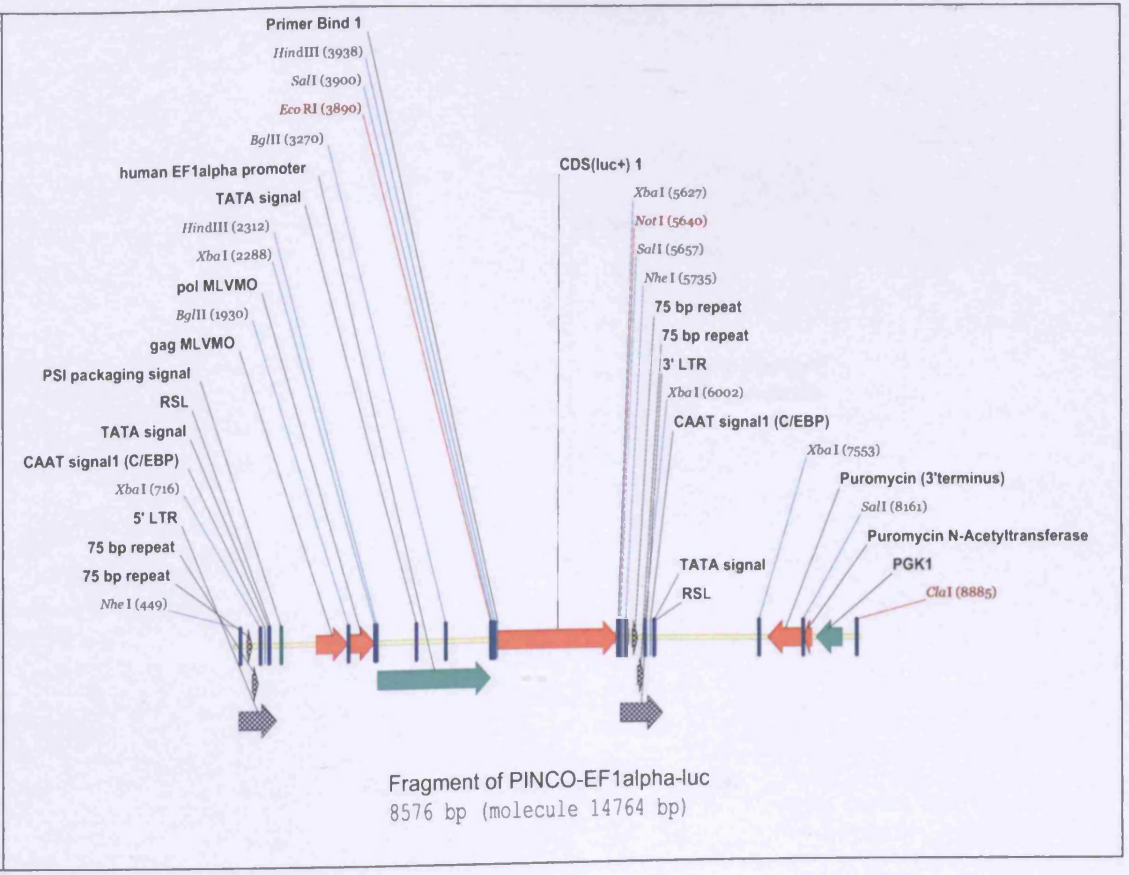
Insert plasmid: pGL3-Basic

Digest: BglII/XmaIII

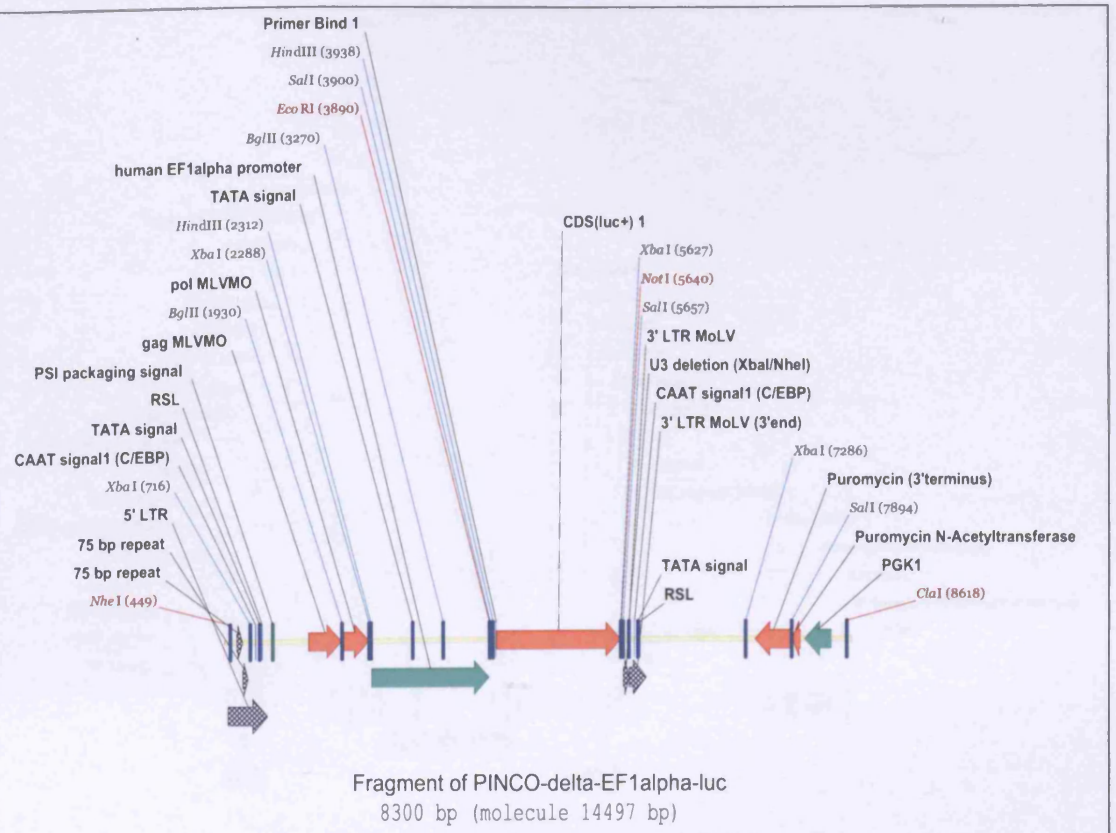
Insert orientation confirmation

Digest: HindIII

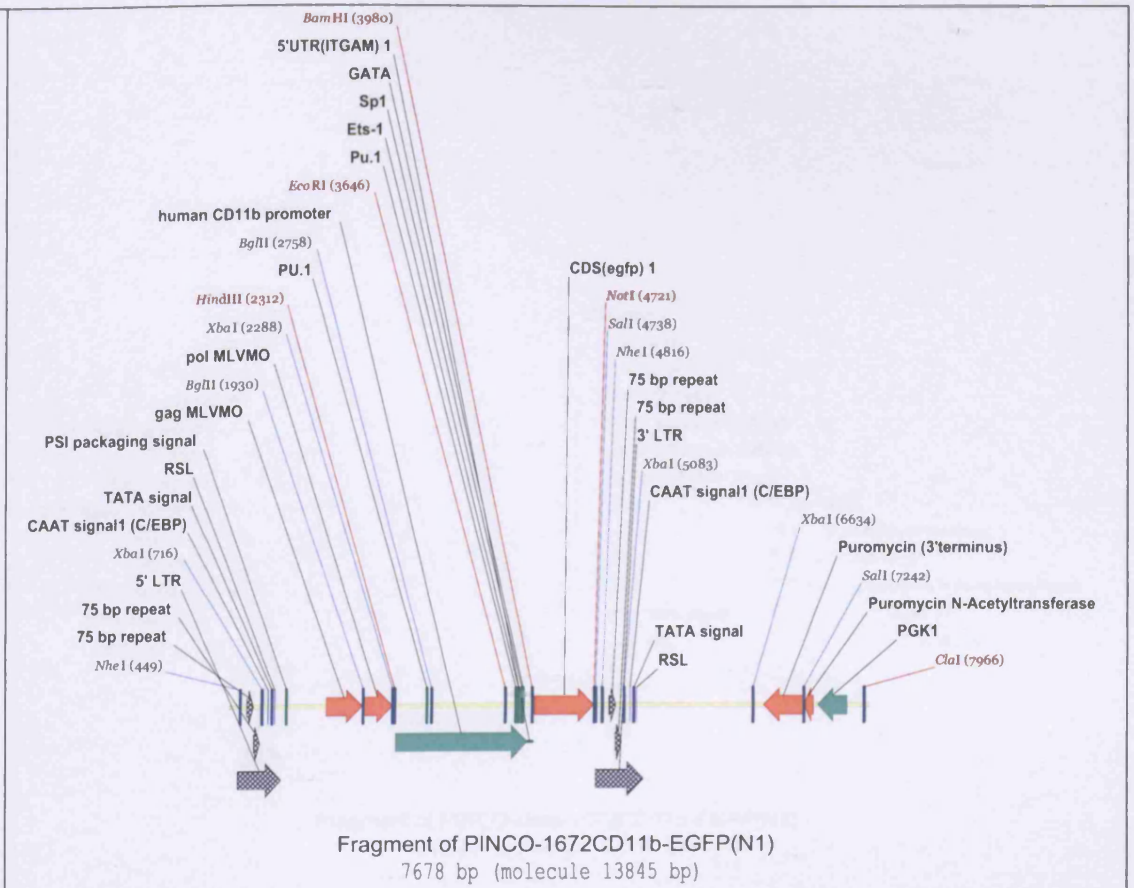
Reference: this thesis



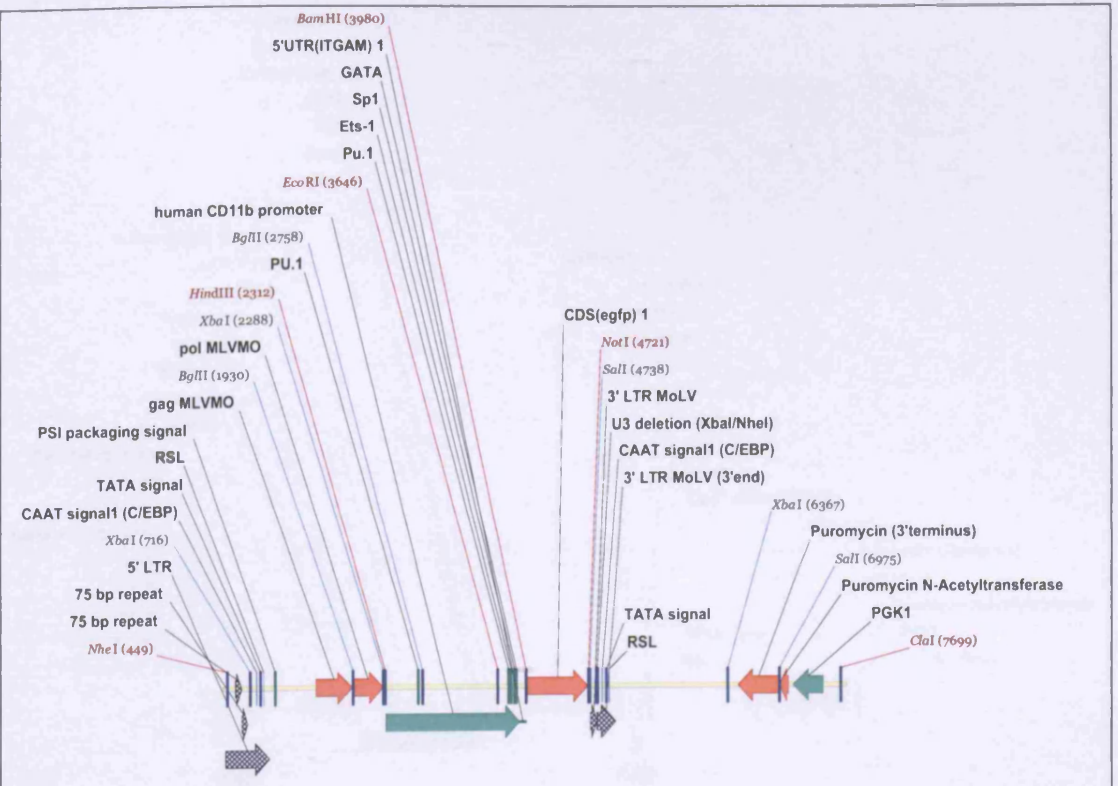
8.5.22 PINCOΔ-1189EF1α-luc

Endogenous Promoter: 5'LTR Exogenous Promoter: EF1α (1189bp) Modification: Enhancer deleted 3'LTR Reporter gene: luciferase Cloning strategy: Backbone plasmid: PINCOΔ-1189EF1α-eGFP Digest: BamHI/NotI Insert plasmid: pGL3-Basic Digest: BglII/XmaIII Insert orientation confirmation Digest: HindIII Reference: this thesis	

8.5.23 PINCO-1672CD11b-eGFP(N1)

Endogenous Promoter: 5'LTR Exogenous Promoter: CD11b (1672bp) Reporter gene: eGFP Cloning strategy: Backbone plasmid: PINCO-Basic-eGFP Digest: XhoI/NotI Insert plasmid: pN1-CMV-1672CD11b-eGFP Digest: XhoI/NotI Insert orientation confirmation Digest: BglII Reference: this thesis	

8.5.24 PINCOΔ-1672CD11b-eGFP(N1)

Endogenous Promoter: 5'LTR Exogenous Promoter: CD11b (1672bp) Modification: Enhancer deleted 3'LTR Reporter gene: eGFP Cloning strategy: Backbone plasmid: PINCOΔ-Basic-eGFP Digest: XhoI/NotI Insert plasmid: pN1-CMV-1672CD11b-eGFP Digest: XhoI/NotI Insert orientation confirmation Digest: BglII Reference: this thesis	

8.5.25 PINCO-1672CD11b-luc(N1)

Endogenous Promoter: 5'LTR

Exogenous Promoter: CD11b (1672bp)

Reporter gene: luciferase

Cloning strategy:

Backbone plasmid: PINCO-Basic-eGFP

Digest: XhoI/NotI

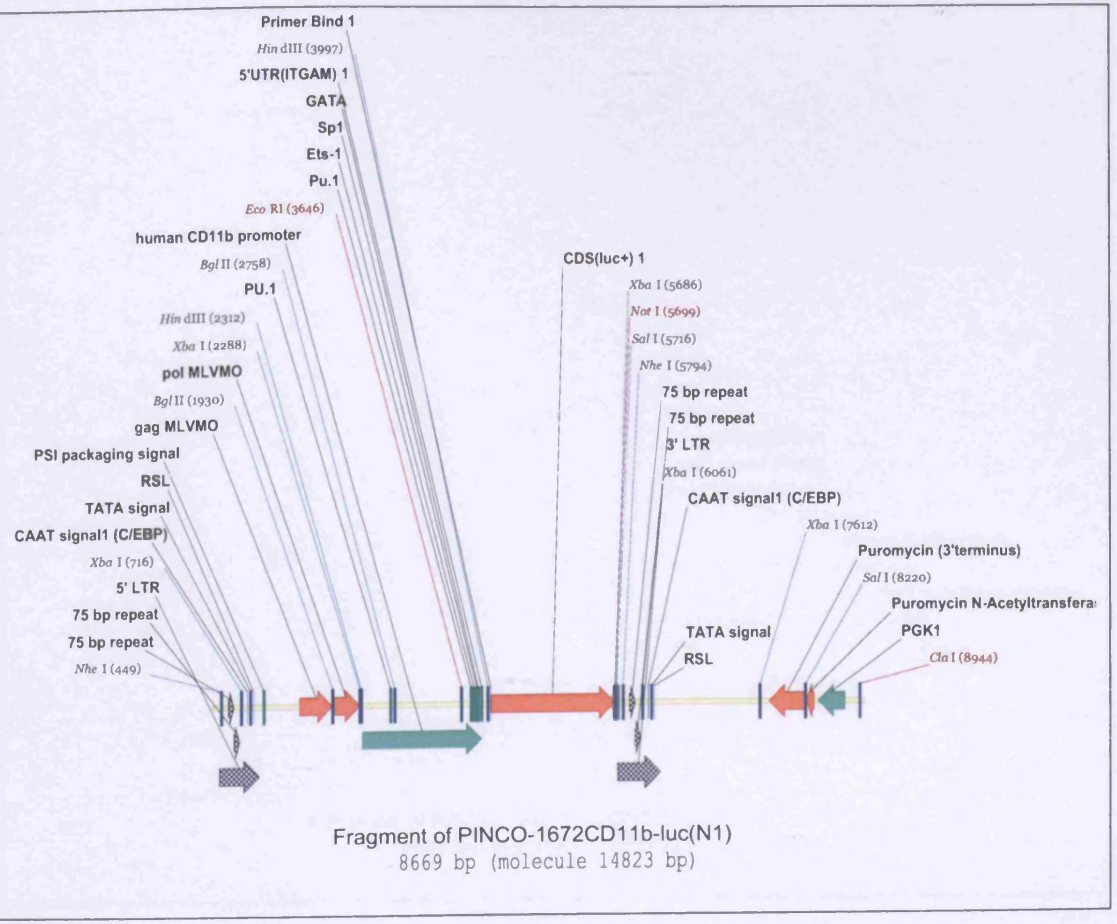
Insert plasmid: pN1-CMV-1672CD11b-luc

Digest: XhoI/NotI

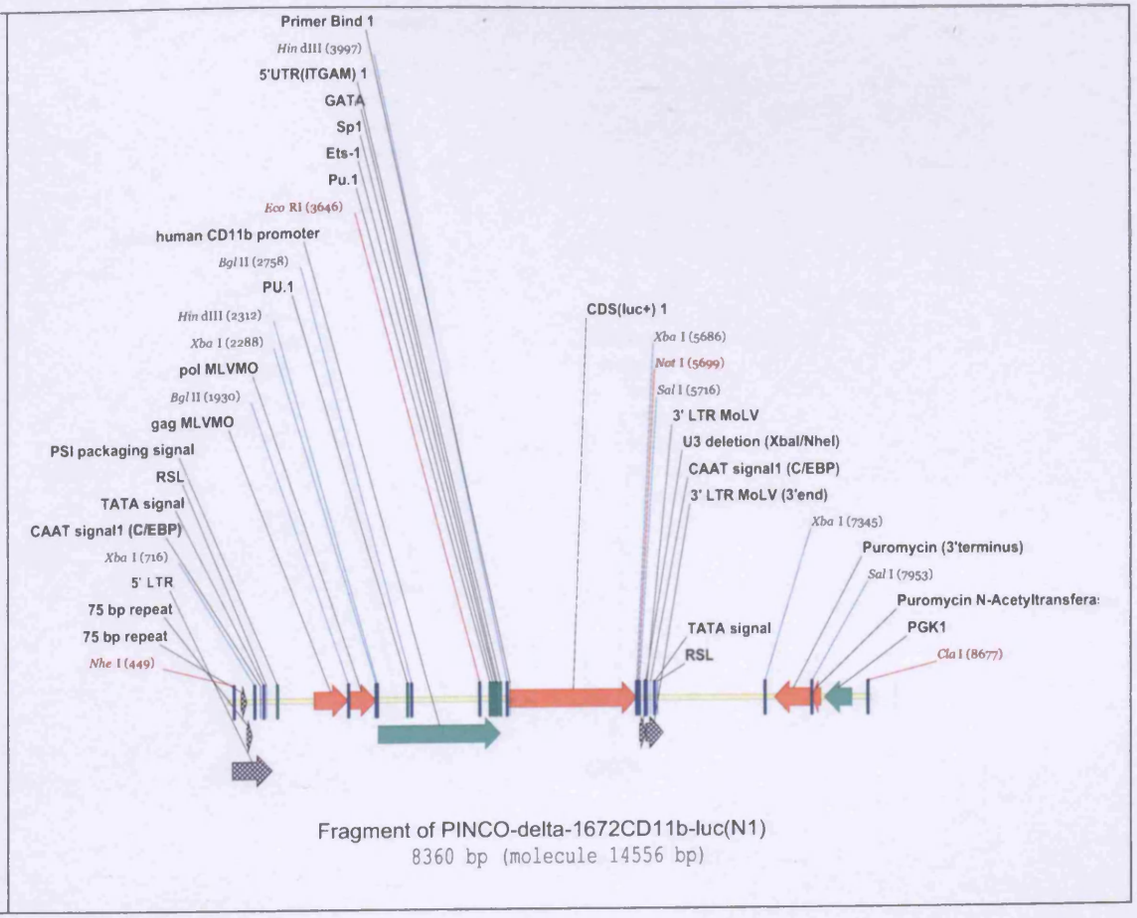
Insert orientation confirmation

Digest: HindIII

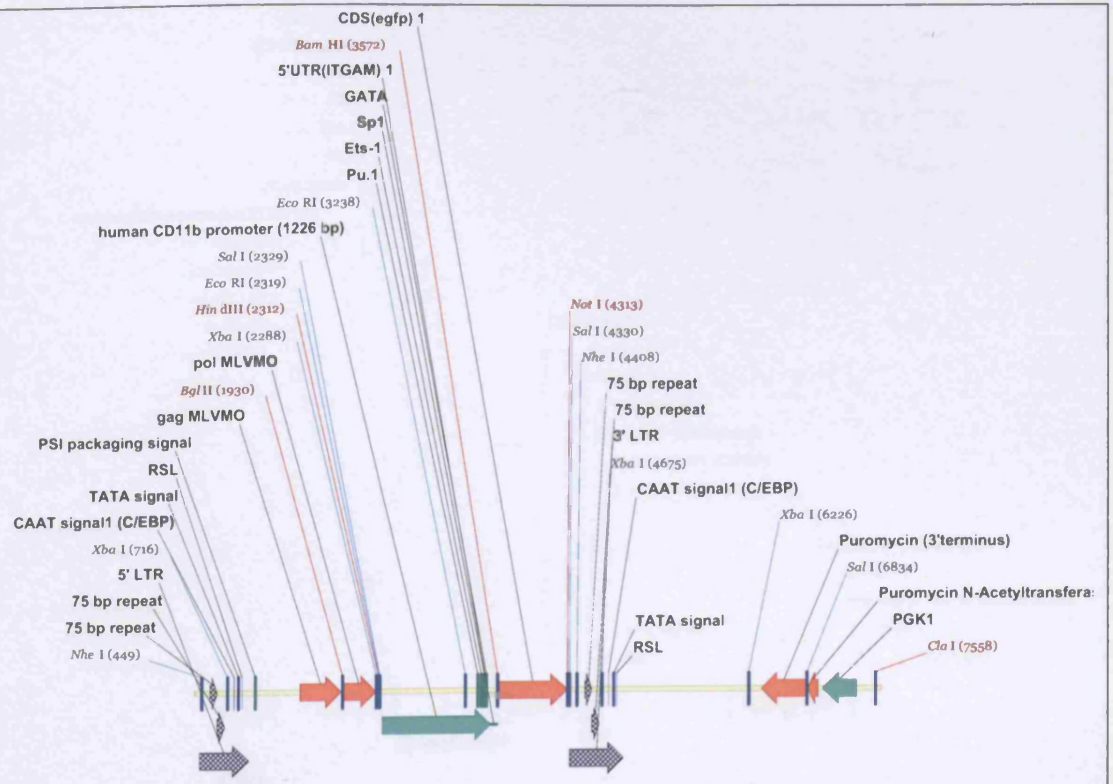
Reference: this thesis



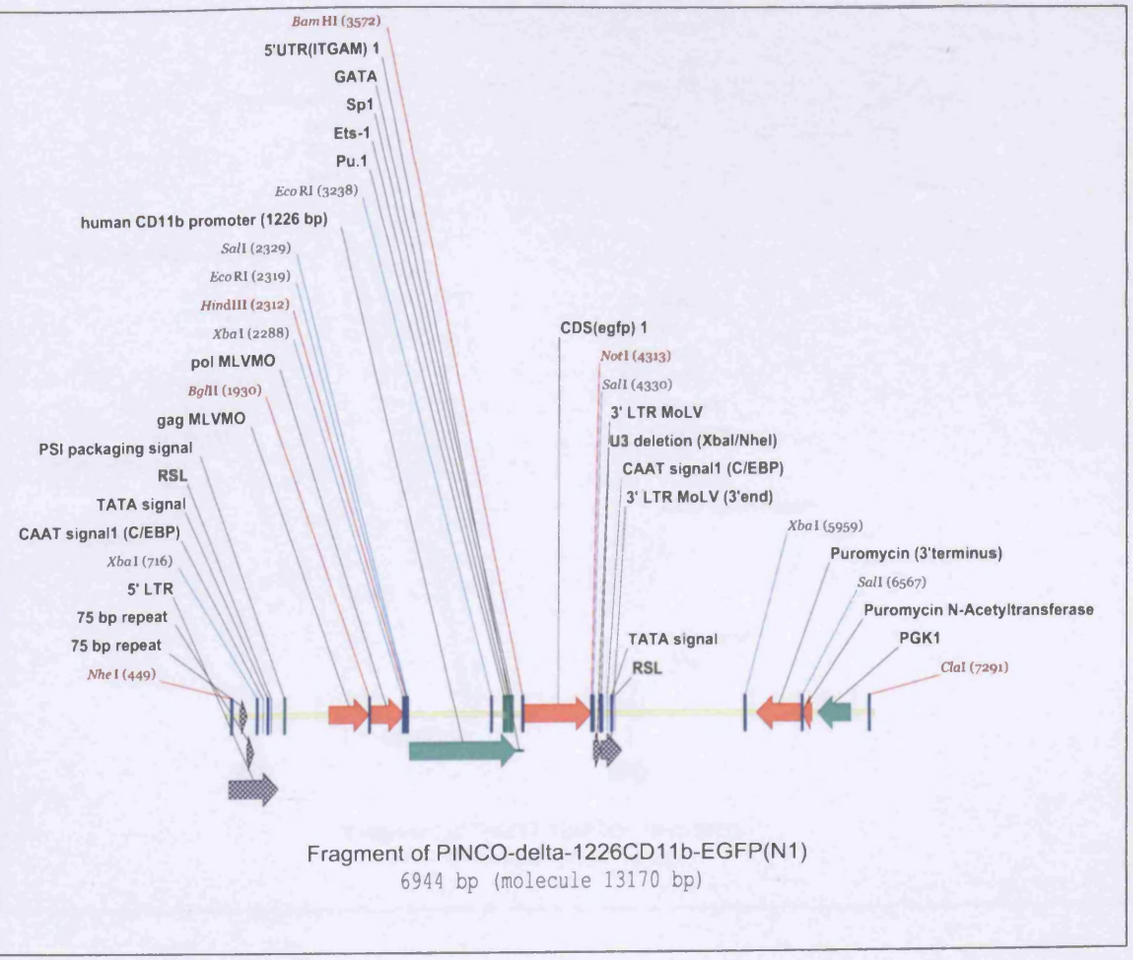
8.5.26 PINCOΔ-1672CD11b-luc(N1)

Endogenous Promoter: 5'LTR Exogenous Promoter: CD11b (1672bp) Modification: Enhancer deleted 3'LTR Reporter gene: eGFP Cloning strategy: Backbone plasmid: PINCOΔ-Basic-eGFP Digest: XhoI/NotI Insert plasmid: pN1-CMV-1672CD11b-luc Digest: XhoI/NotI Insert orientation confirmation Digest: HindIII Reference: this thesis	

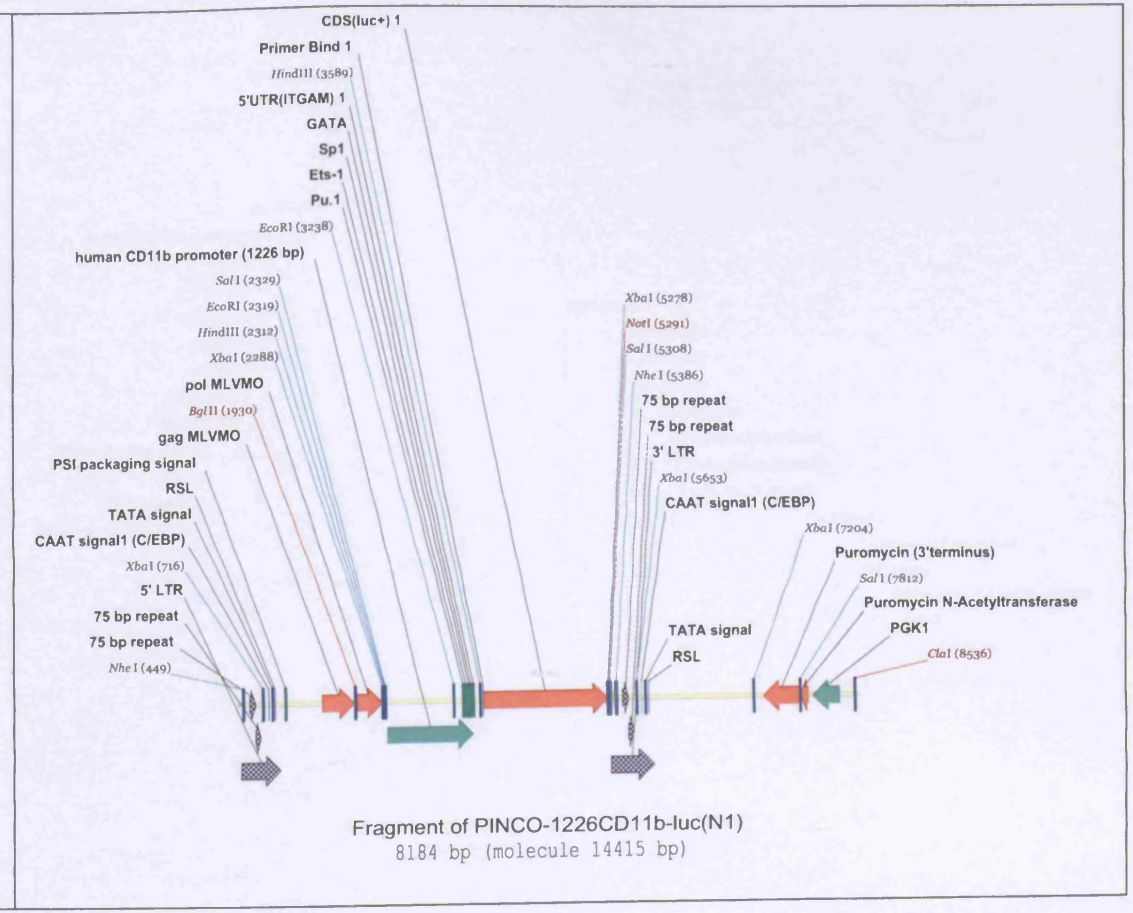
8.5.27 PINCO-1226CD11b-eGFP(N1)

Endogenous Promoter: 5'LTR Exogenous Promoter: CD11b (1226bp) Reporter gene: luciferase Cloning strategy: Backbone plasmid: PINCO-Basic-eGFP Digest: BamHI/NotI Insert plasmid: pN1-CMV-1672CD11b-eGFP Digest: BglII/NotI Insert orientation confirmation Digest: EcoRI Reference: this thesis	

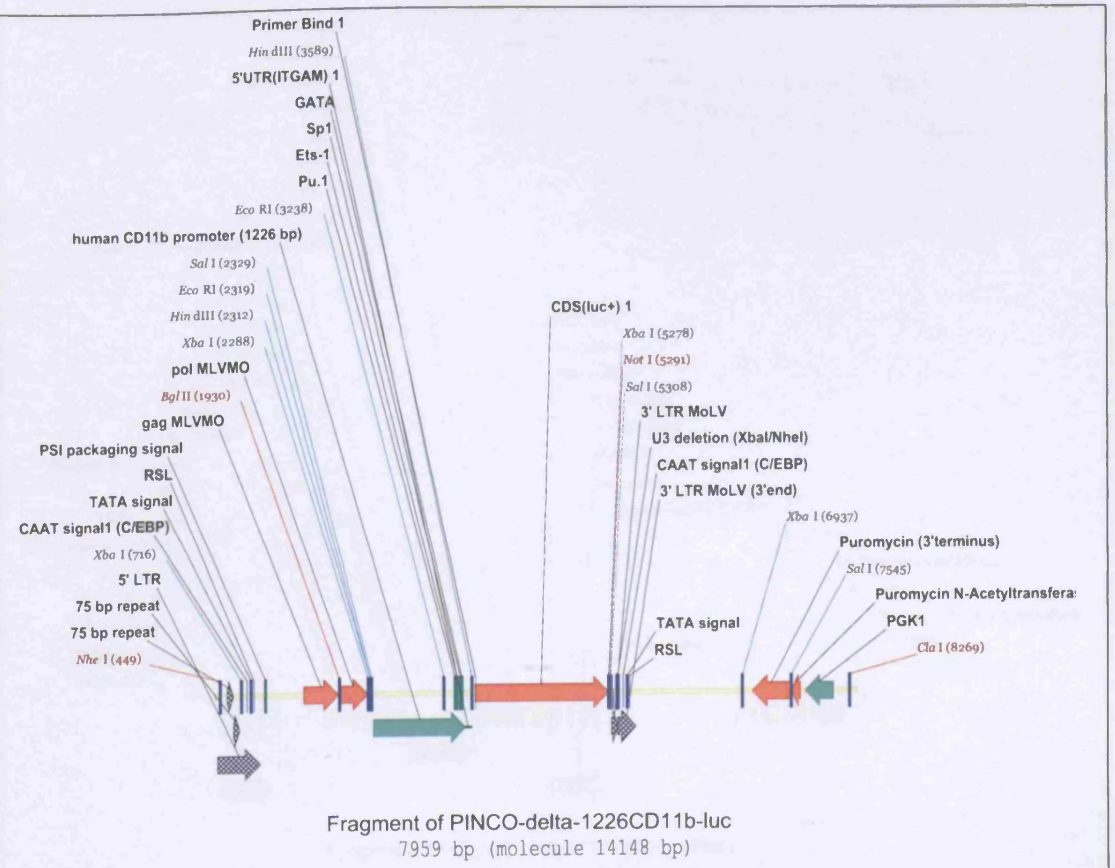
8.5.28 PINCOΔ-1226CD11b-eGFP(N1)

Endogenous Promoter: 5'LTR Exogenous Promoter: CD11b (1226bp) Modification: Enhancer deleted 3'LTR Reporter gene: luciferase Cloning strategy: Backbone plasmid: PINCOΔ-Basic-eGFP Digest: BamHI/NotI Insert plasmid: pN1-CMV-1672CD11b-eGFP Digest: BglII/NotI Insert orientation confirmation Digest: EcoRI Reference: this thesis	

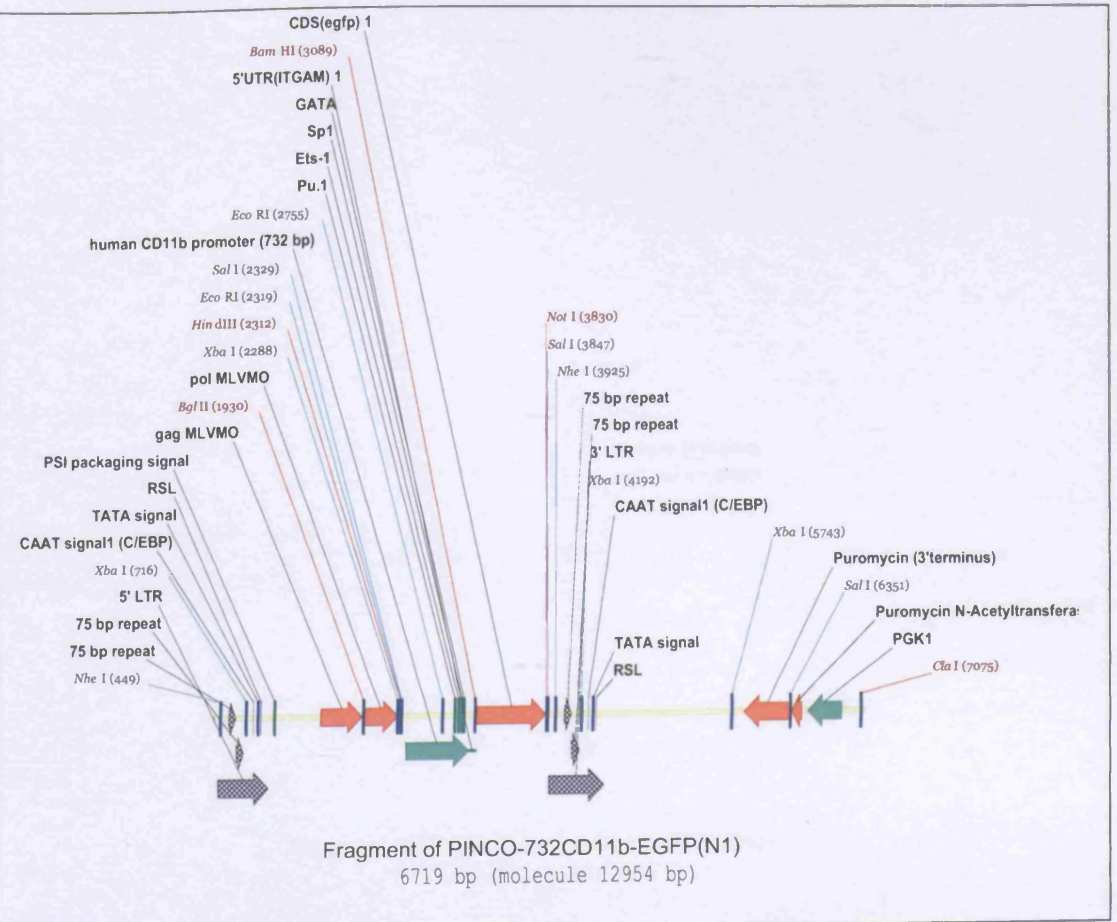
8.5.29 PINCO-1226CD11b-luc(N1)

Endogenous Promoter: 5'LTR Exogenous Promoter: CD11b (1226bp) Reporter gene: luciferase Cloning strategy: Backbone plasmid: PINCO-Basic-eGFP Digest: BamHI/NotI Insert plasmid: pN1-CMV-1672CD11b-luc Digest: BglII/NotI Insert orientation confirmation Digest: EcoRI Reference: this thesis	

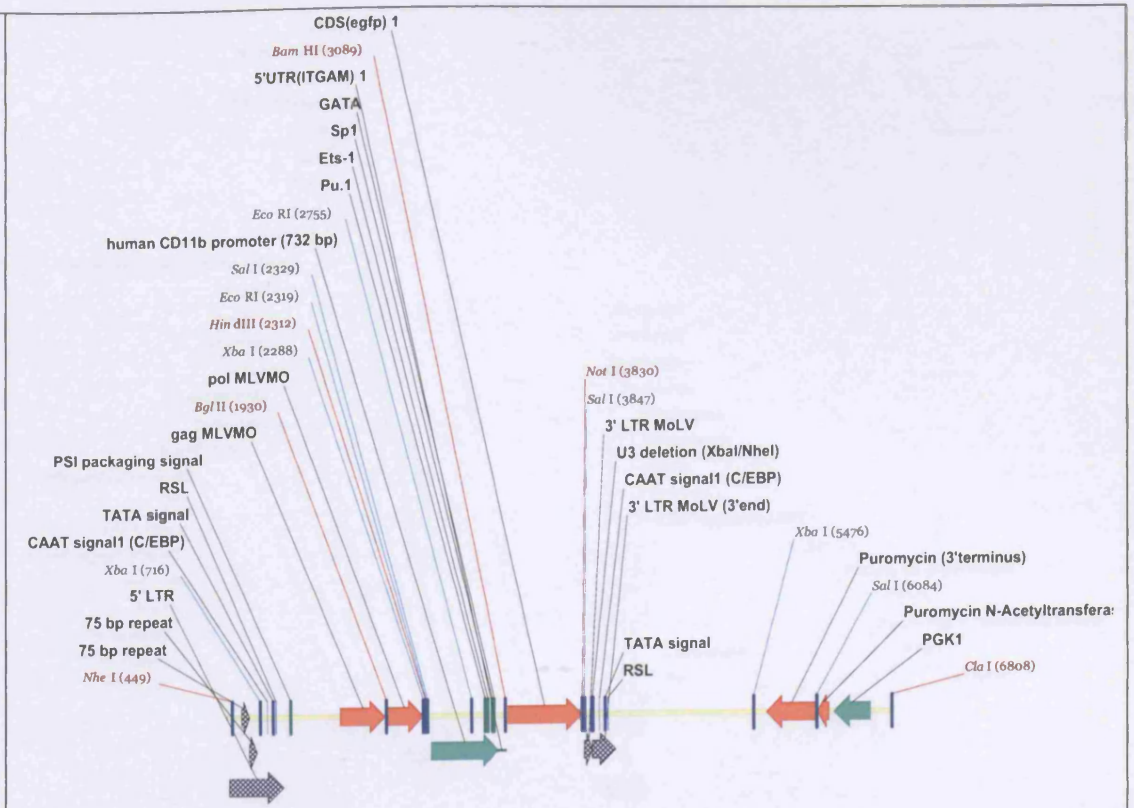
8.5.30 PINCOΔ-1226CD11b-luc(N1)

Endogenous Promoter: 5'LTR Exogenous Promoter: CD11b (1226bp) Modification: Enhancer deleted 3'LTR Reporter gene: luciferase Cloning strategy: Backbone plasmid: PINCOΔ-Basic-eGFP Digest: BamHI/NotI Insert plasmid: pN1-CMV-1672CD11b-luc Digest: BglII/NotI Insert orientation confirmation Digest: EcoRI Reference: this thesis	

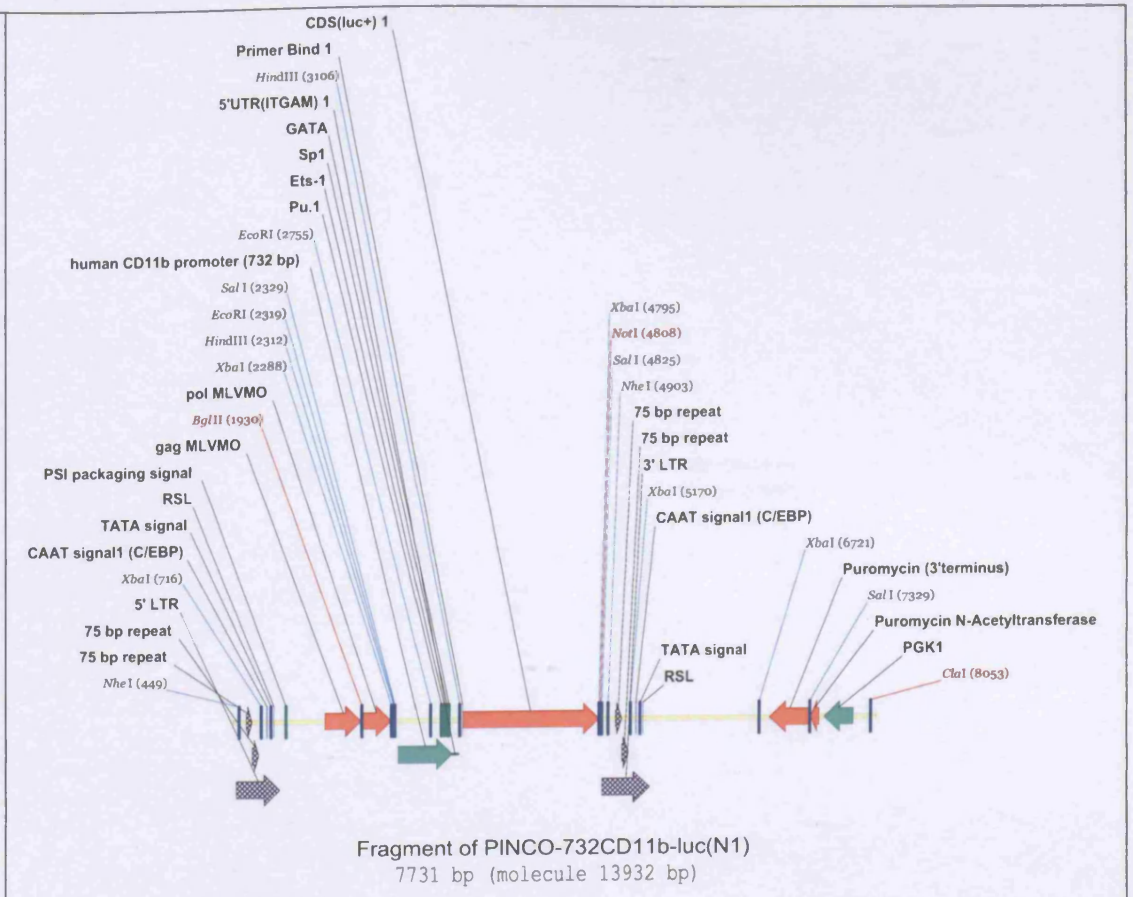
8.5.31 PINCO-732CD11b-eGFP(N1)

Endogenous Promoter: 5'LTR Exogenous Promoter: CD11b (732bp) Reporter gene: luciferase Cloning strategy: Backbone plasmid: PINCO-Basic-eGFP Digest: BamHI/NotI Insert plasmid: pN1-CMV-732CD11b-eGFP Digest: BglII/NotI Insert orientation confirmation Digest: EcoRI Reference: this thesis	

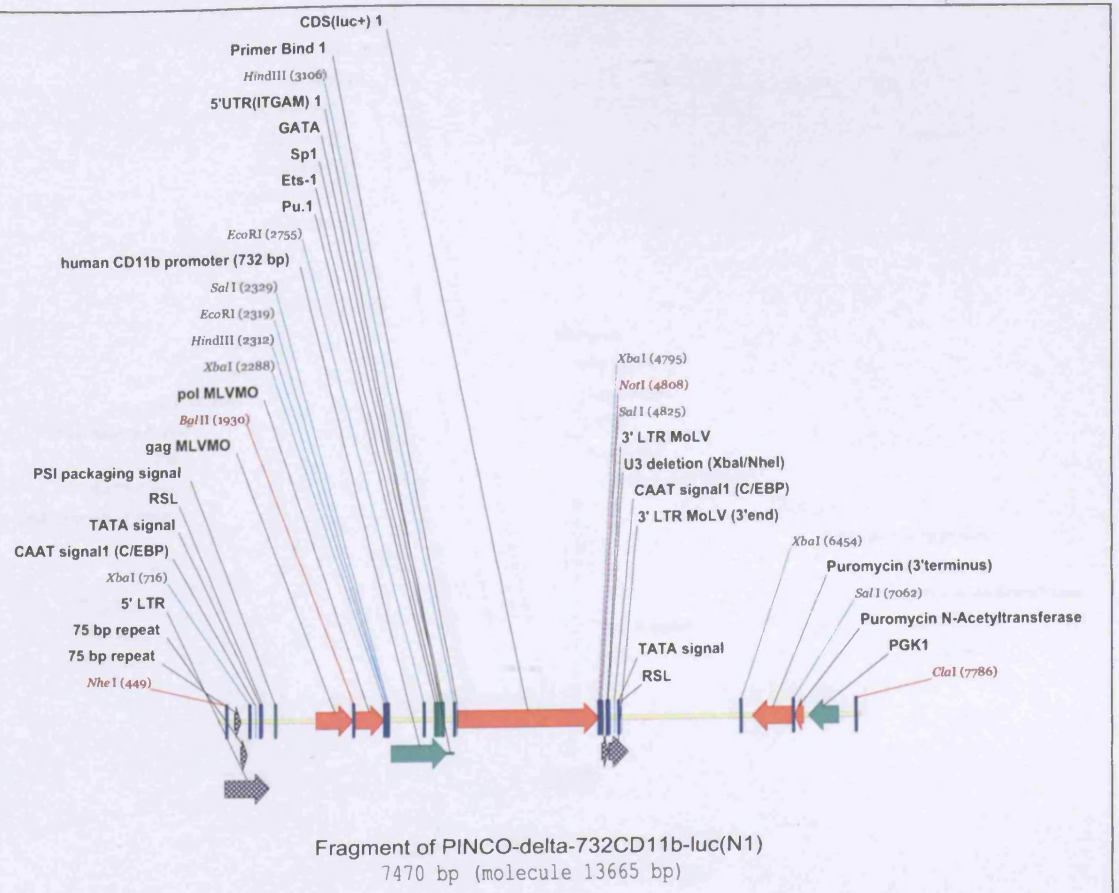
8.5.32 PINCOΔ-732CD11b-eGFP(N1)

Endogenous Promoter: 5'LTR Exogenous Promoter: CD11b (732bp) Modification: Enhancer deleted 3'LTR Reporter gene: luciferase Cloning strategy: Backbone plasmid: PINCOΔ-Basic-eGFP Digest: BamHI/NotI Insert plasmid: pN1-CMV-732CD11b-eGFP Digest: BglII/NotI Insert orientation confirmation Digest: EcoRI Reference: this thesis	

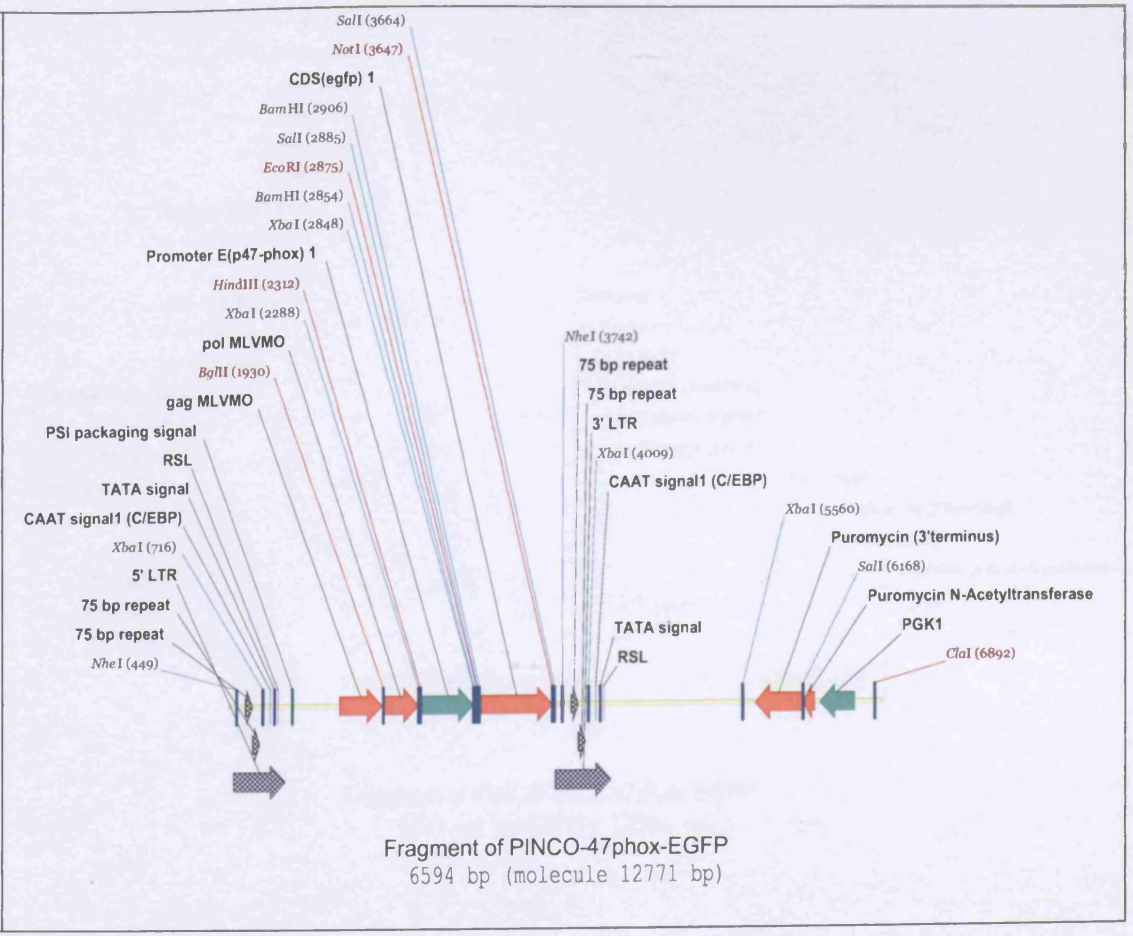
8.5.33 PINCO-732CD11b-luc(N1)

Endogenous Promoter: 5'LTR Exogenous Promoter: CD11b (732bp) Reporter gene: luciferase Cloning strategy: Backbone plasmid: PINCO-Basic-eGFP Digest: BamHI/NotI Insert plasmid: pN1-CMV-732CD11b-luc Digest: BglII/NotI Insert orientation confirmation Digest: HindIII Reference: this thesis	

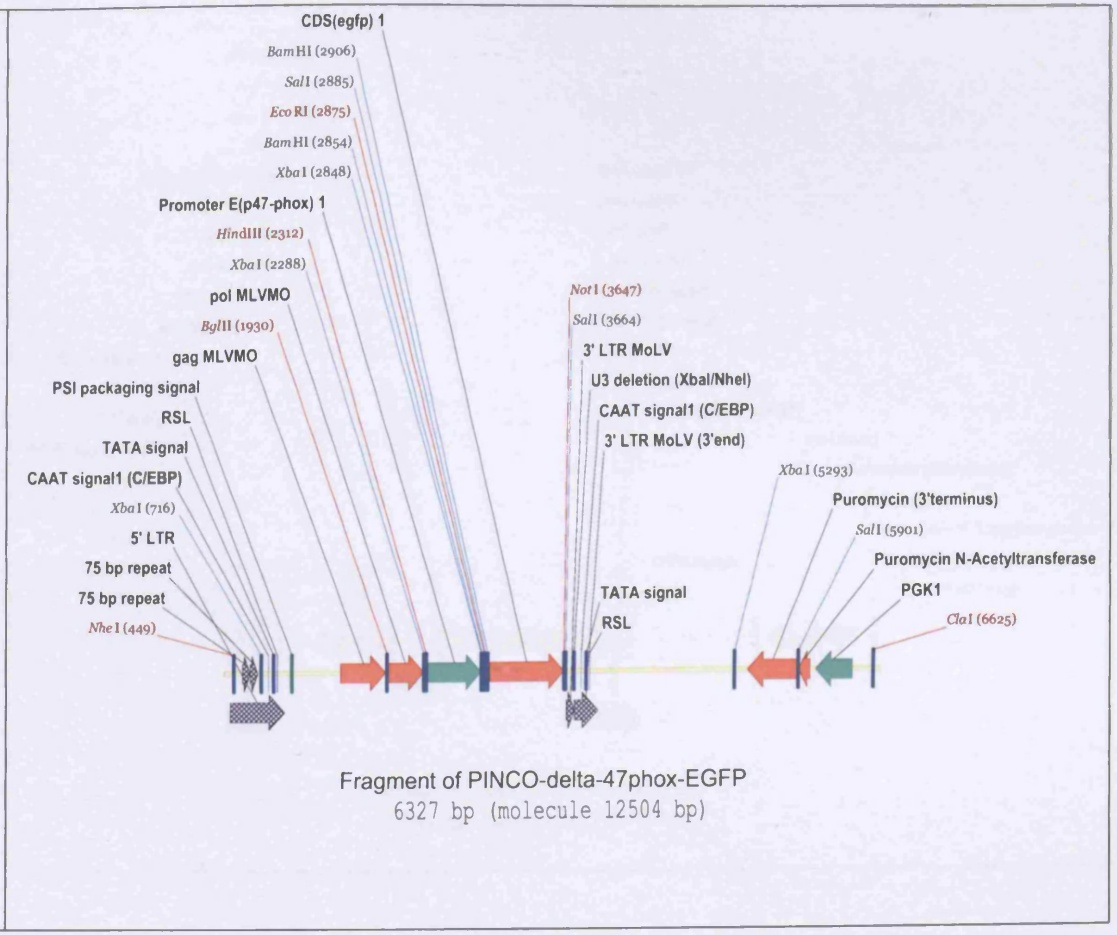
8.5.34 PINCOΔ-732CD11b-luc(N1)

Endogenous Promoter: 5'LTR Exogenous Promoter: CD11b (732bp) Modification: Enhancer deleted 3'LTR Reporter gene: luciferase Cloning strategy: Backbone plasmid: PINCOΔ-Basic-eGFP Digest: BamHI/NotI Insert plasmid: pN1-CMV-732CD11b-luc Digest: BglII/NotI Insert orientation confirmation Digest: HindIII Reference: this thesis	

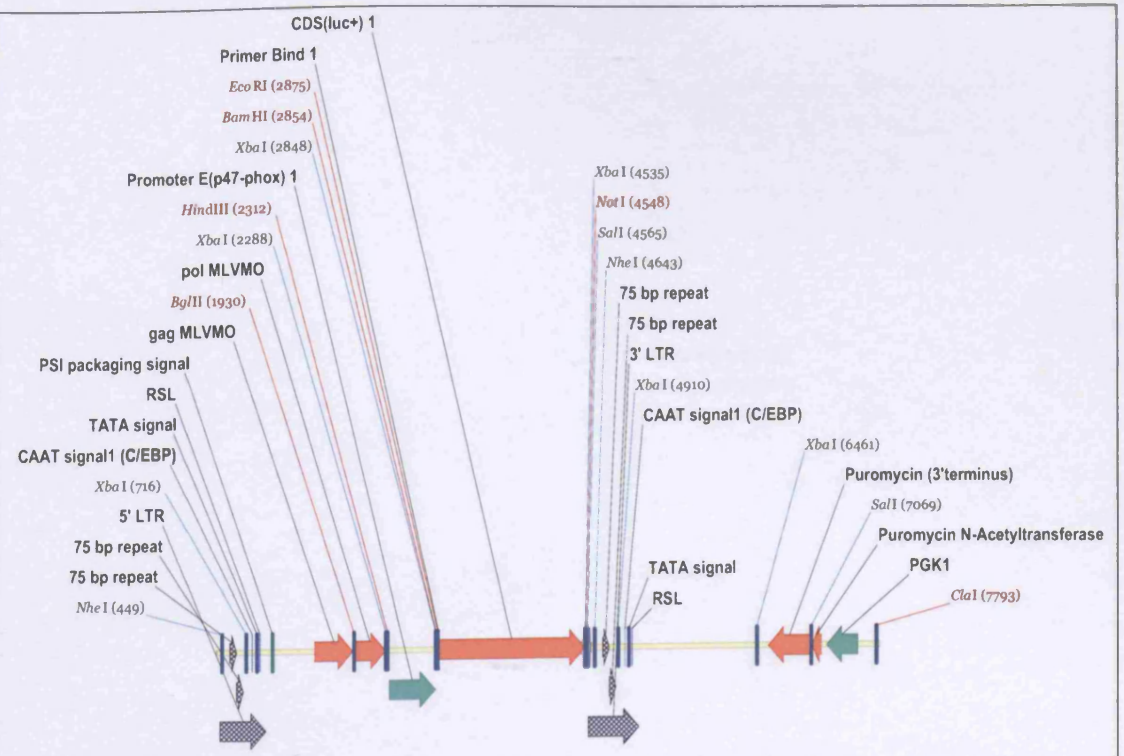
8.5.35 PINCO-p47phox-eGFP

Endogenous Promoter: 5'LTR Exogenous Promoter: p47phox Reporter gene: eGFP Cloning strategy: Backbone plasmid: PINCO-EF1α-eGFP Digest: HindIII/EcoRI Insert plasmid: p47cDNA/p47phox Digest: HindIII/EcoRI Insert orientation confirmation Digest: XbaI Reference: this thesis	

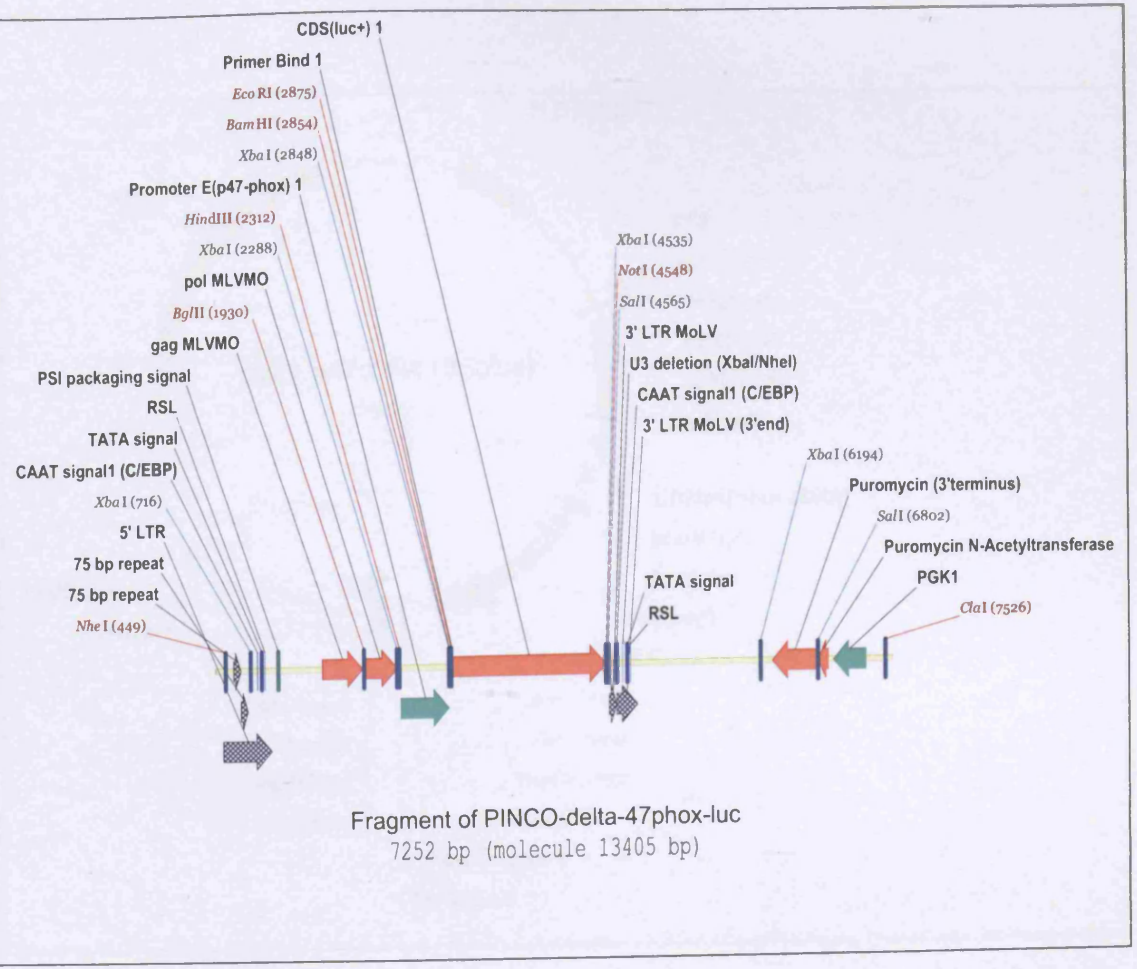
8.5.36 PINCOΔ-p47phox-eGFP

Endogenous Promoter: 5'LTR Exogenous Promoter: p47phox Modification Enhancer deleted 3'LTR Reporter gene: eGFP Cloning strategy: Backbone plasmid: PINCOΔ-EF1α-eGFP Digest: HindIII/EcoRI Insert plasmid: p47/47 Digest: HindIII/EcoRI Insert orientation confirmation Digest: XbaI Reference: this thesis	

8.5.37 PINCO-p47phox-luc

Endogenous Promoter: 5'LTR Exogenous Promoter: p47phox Reporter gene: eGFP Cloning strategy: Backbone plasmid: PINCO-EF1α-eGFP Digest: HindIII/EcoRI Insert plasmid: p47/47 Digest: HindIII/EcoRI Insert orientation confirmation Digest: XbaI Reference: this thesis	

8.5.38 PINCOΔ-p47phox-luc

Endogenous Promoter: 5'LTR Exogenous Promoter: p47phox Modification: Enhancer deleted 3'LTR Reporter gene: eGFP Cloning strategy: Backbone plasmid: PINCOΔ-47phox-eGFP Digest: EcoRI/NotI Insert plasmid: pGL3-EF1α-luc Digest: EcoRI/XmaIII Insert orientation confirmation Digest: XbaI Reference: this thesis	

8.6 Plasmids and subclones with p47phox cDNA and p47phox Promoter

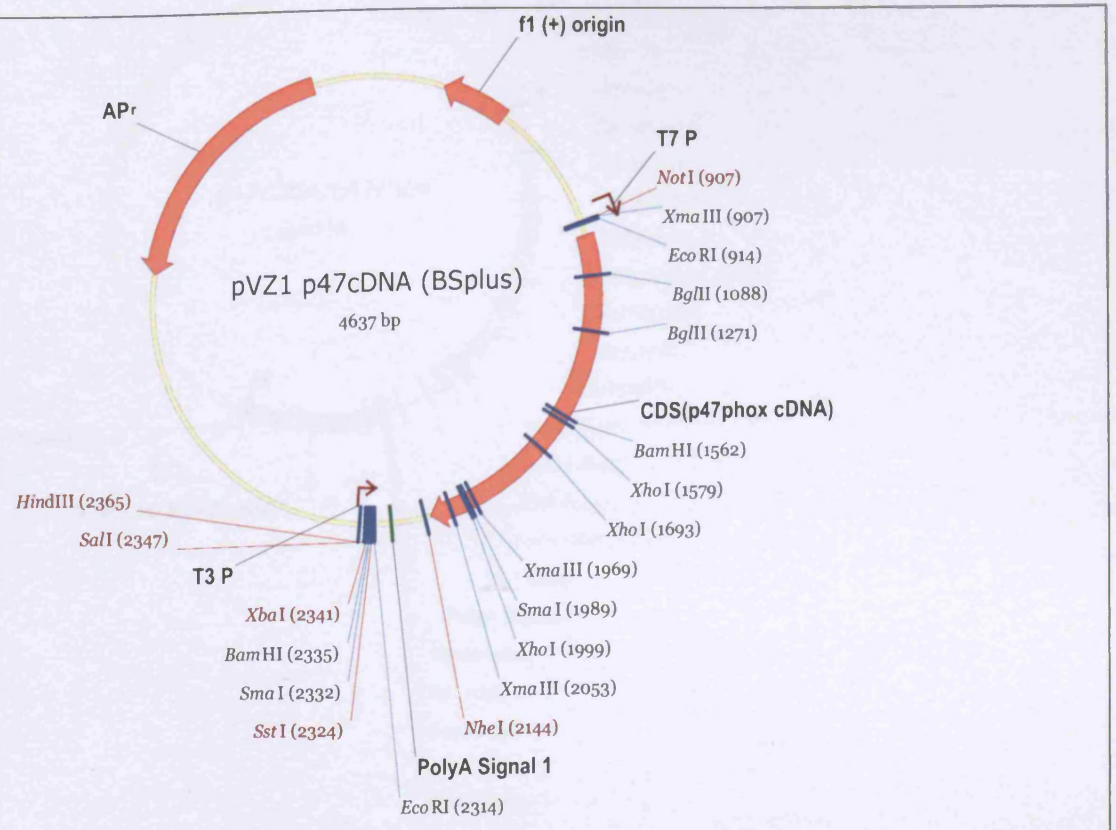
8.6.1 pVZ1-NCF-47k (p47phox cDNA)

(Equal with pVZ1 p47cDNA)

Backbone plasmid: pBlueScript+

Insert: human NCF-47k (p47phox cDNA) flanked by linker sequence

Reference: kind gift from Dr. Adam Rodaway



8.6.2 p47cDNA-p47phox

Cloning strategy:

Backbone plasmid:

pCAT-XRN (8.1.6)

Digest: HindIII/XbaI

Insert plasmid::

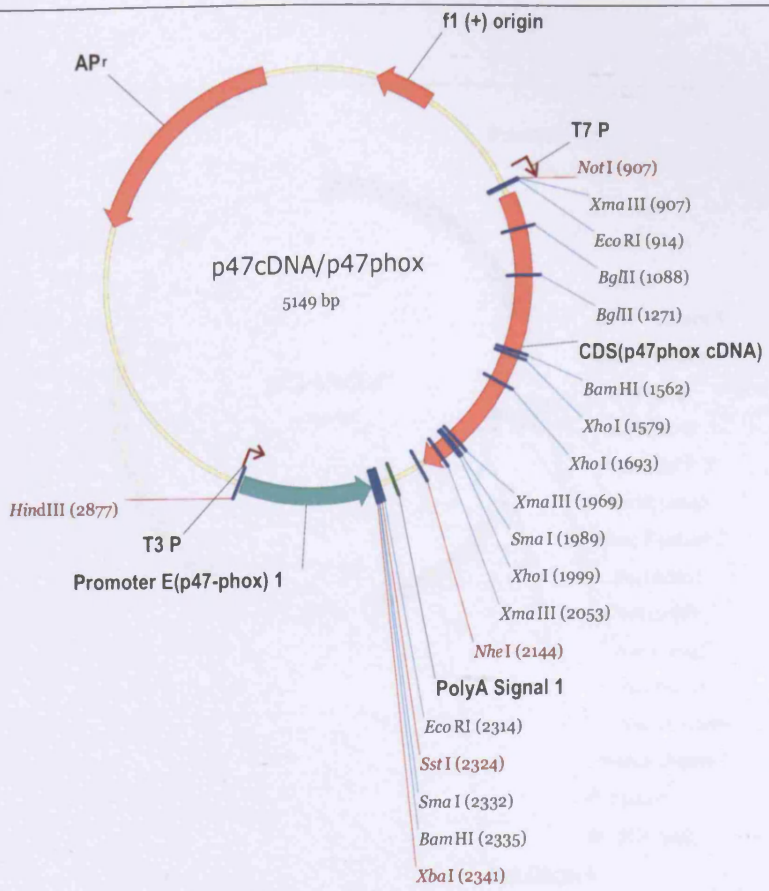
pVZ1-NCF47k (8.6.1)

Digest: HindII/XbaI

cDNA: p47phox cDNA

Promoter: p47phox promoter

Reference: this thesis



8.7 Plasmids and subclones with chimeric Intron

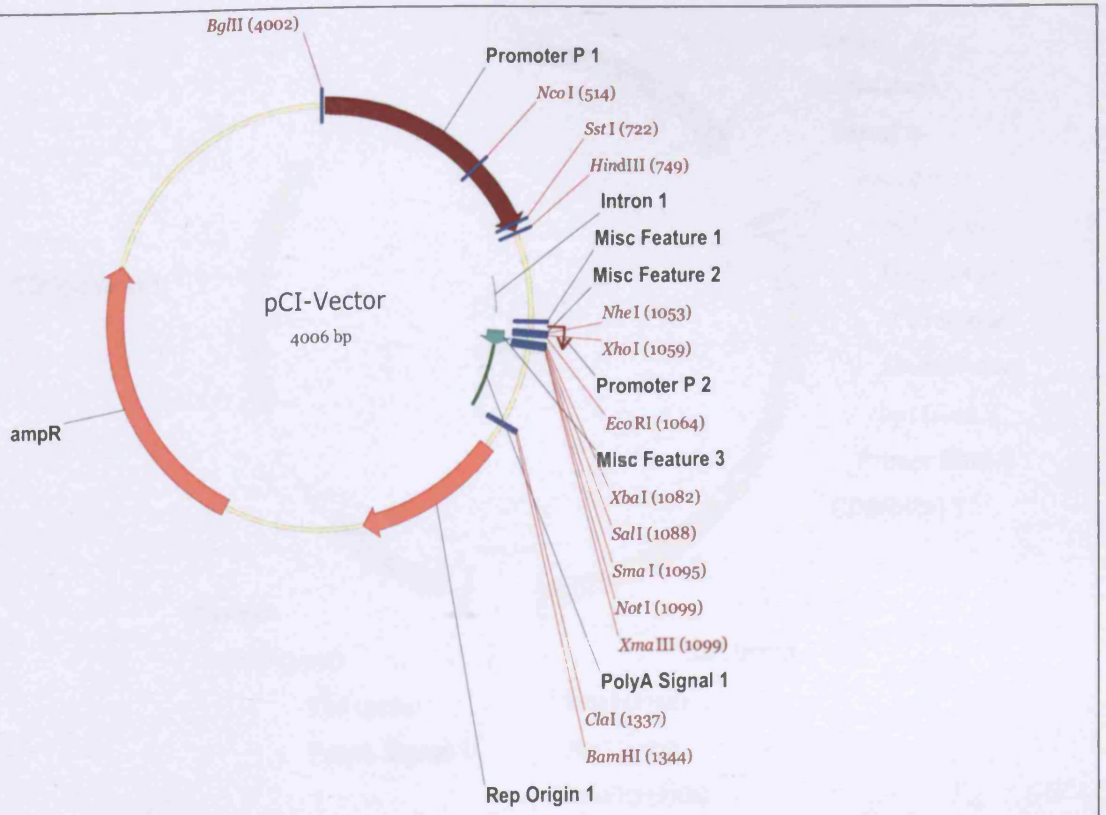
8.7.1 pCI-Vector

Promoter: CMV IE

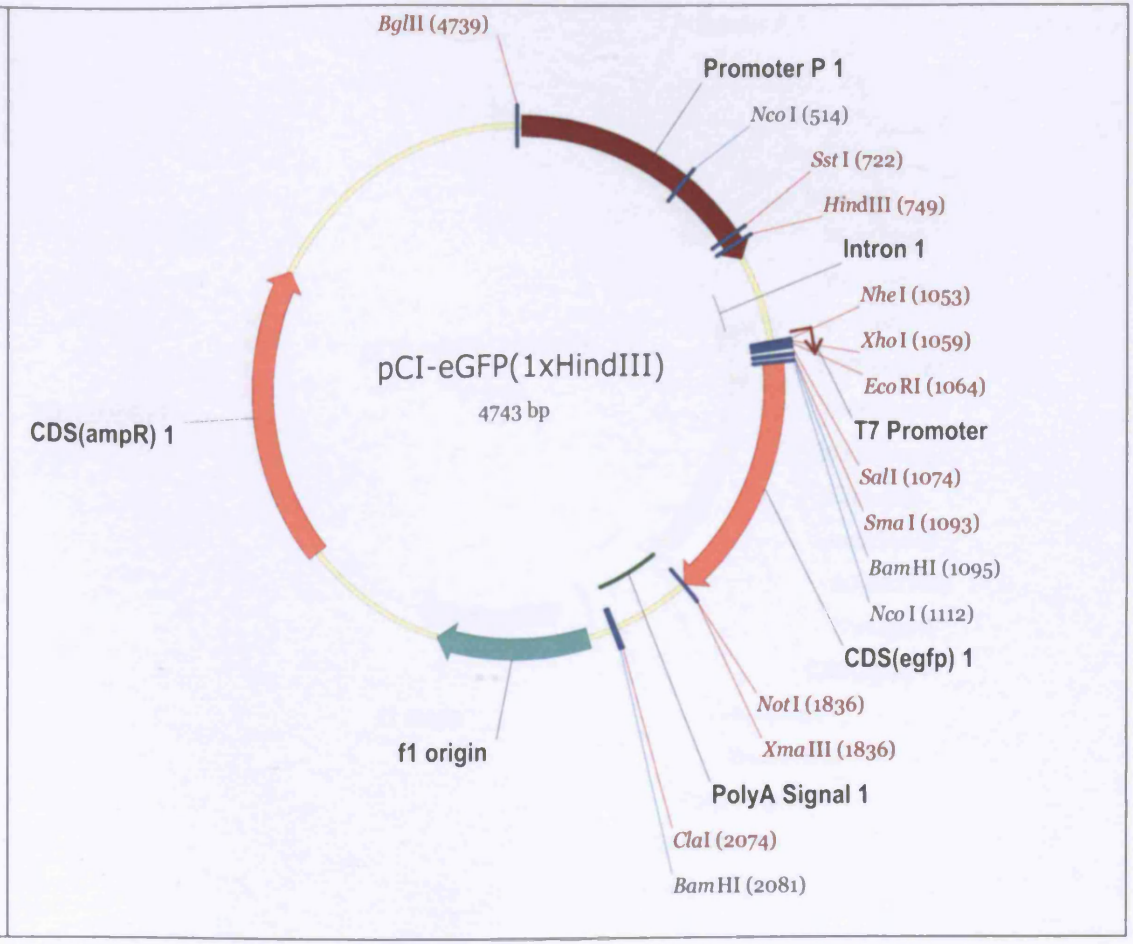
Mammalian Expression vector with chimeric intron downstream of the enhancer/promoter region

Reporter gene: no

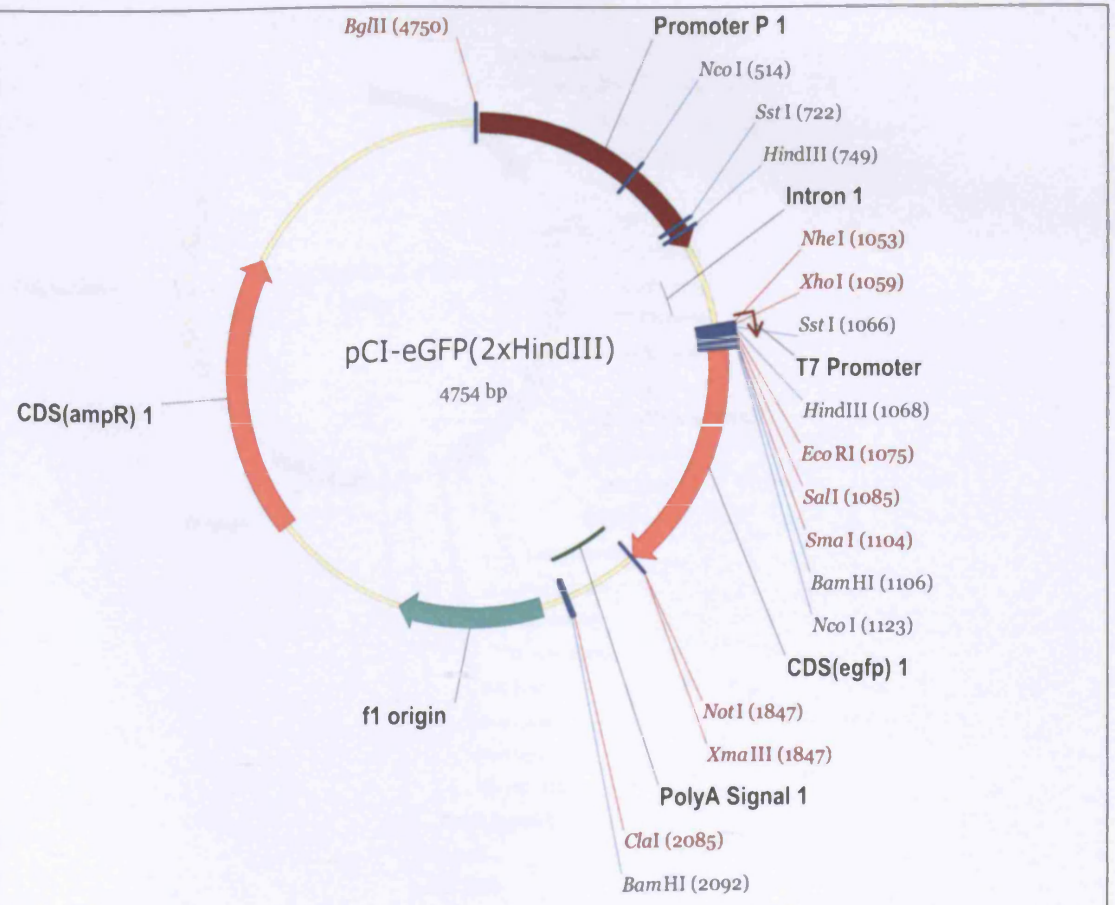
Reference: Promega



8.7.3 pCI-eGFP(1xHindIII)

Promoter: CMV IE Cloning strategy: Backbone plasmid: pCI-Vector Digest: EcoRI/NotI Insert plasmid:: pEGFP-N1 Digest: EcoRI/NotI Reporter gene: eGFP Reference: this thesis	

8.7.4 pCI-eGFP(2xHindIII)

Promoter: CMV IE Cloning strategy: Backbone plasmid: pCI-Vector Digest: XhoI/NotI Insert plasmid:: pEGFP-N1 Digest: XhoI/NotI Reporter gene: eGFP Reference: this thesis	

8.7.5 pCI-47cDNA(1xHindIII)

Promoter: CMV IE

Cloning strategy:

Backbone plasmid:

pCI-Vector

Digest: EcoRI/XbaI

Insert plasmid: pVZ1- NCF-47k

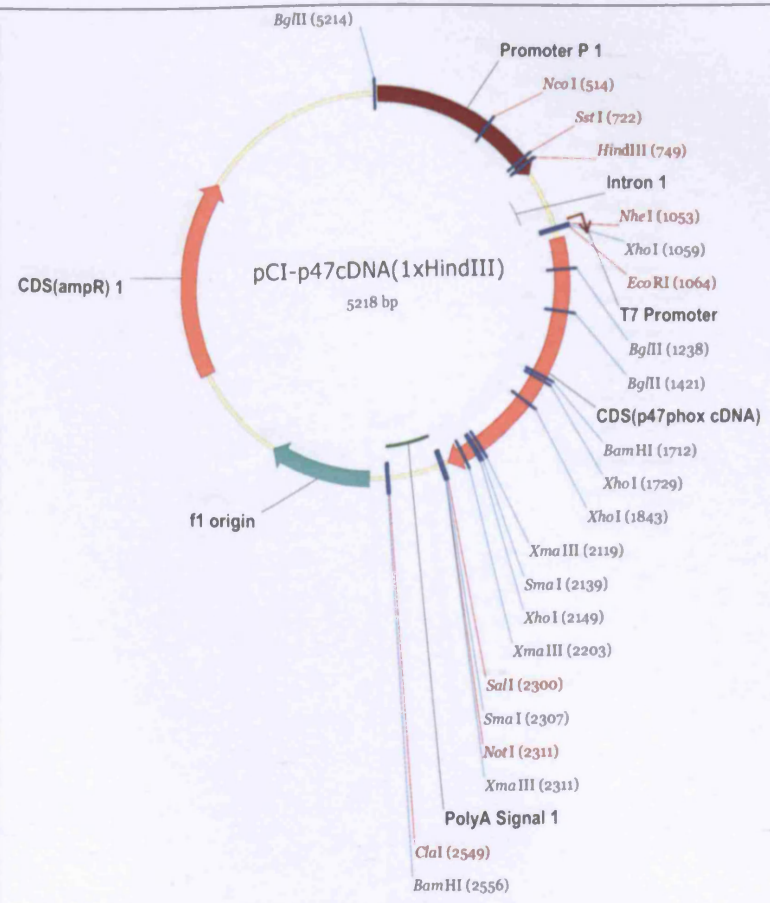
(equal pBS_linkerNCF-47k)

Digest: EcoRI/NheI

Reporter gene: NFC-47k (cDNA)

Reference: this thesis

Equal to pCI-p47cDNA (Schwickerath et al., 2004)



8.7.6 pCI-47cDNA(2xHindIII)

Promoter: CMV IE

Cloning strategy:

Backbone plasmid:

pCI-eGFP (2xHindIII)

Digest: EcoRI/NotI

Insert plasmid::

pCI-47cDNA(1xHindIII)

Digest: EcoRI/NotI

Reporter gene: NFC-47k (cDNA)

Reference: this thesis